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PREFACE

With the completion of the next few paragraphs, Volume 22 of the *Annual Review of Biochemistry* will be ready for the press. We are happy to record that all of the articles previously announced for inclusion in the volume were received. Several of them came to us later than had been anticipated and could only be included by substantial rearrangement of the subject matter. The very late arrivals have been placed in a terminal position and, unfortunately, the authors of such chapters may not always have received adequate opportunity to scrutinize the proofs.

We wish to thank the contributors for the time and care they have given to the writing of these reviews. In many instances, the authors were embarrassed by the magnitude of the subjects to be encompassed and by restriction of the space that was made available to them.

Enhancement of research in many of the fundamental fields of biochemistry has been found to make increasingly severe demands upon space. As a result, the present volume is almost as large as any of its predecessors, despite the transfer of some topics in 1950 to new Reviews. In the present volume we have devoted much more space than hitherto to the subject of vitamins. The number of pages to be so devoted in 1954 will be even further increased.

It is a pleasure to draw attention to the introductory chapter by Professor E. V. McCollum. This is the first of a series in which it is the hope of the Editorial Committee that our elders in biochemistry will give to us through chapters of a historical and philosophical character the benefit of their long years of experience in biochemistry.

We express again our deep gratitude to those who have contributed in authorship to the present volume. We acknowledge also the devoted help of our editorial assistants, and of their associates in the business office. Mrs. Lillian Rutherford has been the principal editorial assistant for this present volume. The subject index was prepared with the generous assistance of Professor Clark Griffin.

We would also thank our printers, the George Banta Publishing Company, for their ever helpful co-operation.

H.J.A. H.S.L.
A.K.B. J.M.L.
H.J.D. G.M.
H.A.S.

TOPICS AND AUTHORS
ANNUAL REVIEW OF BIOCHEMISTRY
VOLUME 23 (1954)

ERRATA

Cumulative Author and Subject Index, Volumes XI to XX
page 185: *for* van Kyke, H. B., *read* van Dyke, H. B.

Volume 20

page 35, lines 6 and 7: *for* cystathione *read* cystathionine
page 221, line 7: *for* Frants & Loftfield *read* Frantz & Loftfield
page 334, line 44: *for* condroitin *read* chondroitin
page 360, line 18: *for* 113,000 gm. *read* 113,000 g
page 363, reference 77: for *J. Biol. Chem.* read *J. Gen. Physiol.*
page 537, line 36: *for* Glucose-6-phosphate + pyruvate + CO_2Mn + TPN phosphogluconate + malate *read* Glucose-6-phosphate + pyruvate

$$+CO_2 \xrightarrow[\text{TPN}]{\text{Mn}} \text{phosphogluconate} + \text{malate}$$

Volume 21

page 154, line 14: *for* 3-, 5-, 17-, and 20-epimers *read* 3-, 17-, and 20-epimers
page 303, line 40: *for* Shepartz *read* Schepartz
page 327, reference 35: *for* Shepartz, B., *read* Schepartz, B.
page 488, footnote 4: *for* Karrer (89) *read* Karrer (90)
page 489, line 29: *for* Otto Isler (90) *read* Otto Isler (89)
page 492, reference 94: *for* Tuss, C. D. *read* Buss, C. D.
page 650, line 41 and 42: *for* Watson & Abbott *read* James & Abbott
page 681, reference 179: *for* Watson, J. G., *read* James, G. W.

Author Index

page 742: *insert* James, G. W., 650
page 755: *insert* Schepartz, B., 303, 315
page 756: *delete* Shepartz, B., 303, 315
page 760: *delete* Watson, J. G., 650

Volume 22

Author Index
for Richards, J. C. *read* Rickards, J. C.
for Brown, L. N. *read* Brown, L. H.

CONTENTS


E. V. McCollum
1951

PREFATORY CHAPTER

MY EARLY EXPERIENCES IN THE STUDY OF FOODS AND NUTRITION

By E. V. McCollum

The Johns Hopkins University, Baltimore, Maryland

This chapter was written at the request of the Editorial Committee. Among the suggestions which the Editor offered as to the kind of chapter which would be acceptable was one to the effect that the writer might prepare ". . . an autobiographical sketch in which he describes his own experiences as a student, a teacher, and an investigator." It is this suggestion that has been followed. These reminiscences will be limited to my educational experiences and to the decade of 1907 to 1917, the period of my earliest experiences as a nutrition investigator. This was a decade of special importance for clarifying the ideas of workers in this field as to how to use animals effectively for discovering the existence of hitherto unsuspected nutrients, a task for which chemical procedures alone were inadequate. It was the decade when the initial successes were achieved in determining, in individual naturally occurring food substances, and in some mixtures of foods, the nature of the chemical deficiencies which limited their ability to support physiological well-being in an animal.

As a youth working on a Kansas farm it appeared to me that among all the people I saw, the life of the country doctor, with his many human contacts and general esteem, was the most desirable. I early resolved to study medicine. This resolution I kept through my high school years and my first year at the University of Kansas, in 1900. In my second year I listened with delight to the lectures of Dr. Edward Bartow on elementary organic chemistry and soon made up my mind that the chemistry of organic substances was the field in which I wanted to become proficient and to devote himself.

On completing the first course I proceeded at once to synthesize organic compounds, using the German edition of Gatterman's *Praxis;* I prepared in succession the forty-eight kinds of substances there described, and purified them as directed. Dr. Bartow taught me to use his copy of *Beilstein*. When I reached the synthesis of quinoline, by the method of Skraup, I had my first inspiration in research. As Dr. Bartow's lecture table assistant I had access to the store rooms, where I had seen eight bottles of substituted anilines. It occurred to me that if each of these substances were heated with sulfuric acid and glycerol, there would be obtained instead of quinoline, which resulted from the use of aniline, a series of derivatives of quinoline. I made a list of the names and formulas of the chemicals in these bottles, and then looked in *Beilstein* to see if any of them were described. I found none and told Dr. Bartow about what I had done and would like to do. He said the derivatives of

aniline belonged to Professor E. C. Franklin, and that I should not use them without his permission. I took up this matter with Dr. Franklin, who taught me my first course in quantitative analysis, and my first course in physical chemistry, and he said he was done with them and that I was welcome to them. So I synthesized the new quinoline derivatives, and purified and analyzed them according to the directions of Dr. Bartow. We published the results under joint authorship.

In 1903 I received my A.B. and in 1904 I was awarded the M.A. Degree at Kansas University. For my thesis I presented the data which the late J. Arthur Harris and I secured in a study which we made of the composition of the gas mixture present in the hollow stems of the large water lily *Nelunbo lutea*, which was then very abundant in a lake not far from Lawrence. Harris was a botanist of note and the closest personal friend of my undergraduate years. We camped for a week and collected samples of the gas morning, noon, evening, midnight, and before sunrise, and secured samples on cloudy days as well as sunshiny ones. We never published our data.

While at Kansas University I gained credit in sixteen courses listed by the department of chemistry, and in September, 1904, I began to study under Professor Treat B. Johnson in the Sheffield Scientific School of Yale University. He was then just outgrowing his master, Professor Henry Lord Wheeler, who was still in charge of organic chemistry there. I devoted two years to syntheses in the pyrimidine series.

During these years I shared an apartment with Philip H. Mitchell, a student in physiological chemistry, who became head of physiology at Brown University. From him I heard much of the activities in the laboratory of Professor Mendel. A highly important personal relation was soon established between Samuel H. Clapp and myself. He was working in organic chemical research under Dr. Wheeler but soon became associated with Dr. Thomas B. Osborne, the eminent student of proteins of vegetable origin, at the Connecticut Agricultural Experiment Station in New Haven. Sam was employed by Osborne to apply the newly described "ester method" of E. Fischer, to the determination of the amounts of different amino acids yielded by different proteins on acid hydrolysis. He was a young man of unusual ability and at at once undertook to learn all he could about the chemistry of amino acids. We frequently dined together, and he often spent an evening with me. On these occasions he gave me a course of instruction on what he had learned about these substances. To my great interest he introduced me to the investigations of Miescher, Ritthausen, Kossel, E. Fischer, Abderhalden, and Osborne.

Upon entering post-graduate study in organic chemistry at Yale I was given a key to the private library of Professor Wheeler and at once began a program of examining his scientific journals. I would take down in succession the volumes of a series of journals and turn every page, leisurely, until I came upon a title which interested me. Then I would read carefully the introduction, in which the writer gave some account of previous investigations bear-

ing on his problem, and stated his objective and plan of experiment. I would then examine his experimental data and study the conclusions which he drew from them. Before proceeding further in the volume I would reflect on what I could do in order to shed further light on this problem. Of course these efforts were not very productive of new ideas, but I enjoyed acquiring detailed information about the properties of many substances and their chemical behavior. I formed reading habits which familiarized me with the manner in which mature men applied themselves to scientific investigations. In this way I saw all of the pages of Liebig's *Annalen*, the *Berichte*, and other chemical journals, and went afield to examine the volumes of Pflüger's *Archives*, the *Zeitschrift für physiologische Chemie*, *Biochemische Zeitschrift*, and *Comptes rendus*. This practice of examining old journals was for me an excellent one since it gave me a clearer perspective of the historical development of organic and biochemical investigations than I could have secured by any other course open to me at that time.

Early in 1906 Clapp gave notice to Dr. Osborne of his intention to give up his position on August 1st and go to Germany for study. Dr. Osborne discussed with Dr. Johnson the matter of finding a man to take Clapp's place. Since I had completed the work which was to be submitted for my dissertation for the Ph.D. degree and could soon finish writing it, it was arranged that I should begin work in Dr. Osborne's laboratory on April 1st and help Clapp, before he left, with the chemical work on hand from previous ester distillations and go through with him the preparation of the esters and a distillation operation. Accordingly I had the good fortune to spend four months with an expert in amino acid chemistry.

Throughout the spring and summer I was hopeful of finding a position in a university where I could teach organic chemistry and try my hand at independent research. No suitable opening came to our notice during that period, so on October 1st I entered the laboratory of Professor Mendel for a year of study of physiological chemistry. I had received the Ph.D. degree in June.

Throughout the academic year 1906–1907 I heard the lectures of Mendel and F. P. Underhill and spent the days in the laboratory gaining familiarity with the analytical methods applicable to biochemical work. I also attended the courses given by Dr. Chittenden on toxicology and on nutrition. The last was a subject in which he was deeply interested. He was a firm believer in the merits of abstemiousness in protein consumption, for which regimen he gave us what seemed to be convincing reasons. It is interesting to note that during this year, so far as I can recollect, there was no mention of beri-beri, scurvy, rickets, or pellagra, by three distinguished teachers of physiological chemistry. Mendel called our attention to the recent experiments of Willcock & Hopkins illustrating the supplementary value of tryptophan for zein, and differences in the amino acid content of proteins, so far as this was known, were discussed from the nutritional interest which they aroused. His instruction was based to a great extent on the researches of Voit, Pettenkofer, Rubner, Atwater, Lusk, and Pavlov, but everything known about metabolic

processes was set before his students. At that time no one anywhere surpassed Professor Mendel in thoroughness of instruction in biochemistry. This is supported by the long list of his students who became professors in this field in universities and came to distinction as teachers and investigators.

In the spring of 1907 Dr. Mendel showed me a letter from Professor E. B. Hart, who, the year before, had succeeded Dr. S. M. Babcock as head of agricultural chemistry at the College of Agriculture of the University of Wisconsin. He inquired for a young man trained in biochemistry who was interested in the study of animal nutrition. Mendel thought the opportunity a good one for me, and since I still had no prospect of securing the kind of position I wanted in organic chemistry, I accepted, after corresponding further with Hart. I entered on my duties in midsummer 1907.

Professor Hart explained to me the plan of his famous experiment with cows restricted to rations derived from single plant sources: the wheat, corn (maize), and oat plants respectively. The experiment had been suggested by Dr. Babcock and was a distinct departure from animal feeding studies of the past. The rations included all parts of the plant except the root, and the parts of the plant were included in such proportions that the entire ration for each group of animals had the same composition as shown by the "Official" method of chemical analysis. A control group received food derived from all three plants, for the purpose of determining whether variety in source of nutrients was of physiological importance (1).

Heifers of 350 to 400 pounds weight had been placed on these rations about a year previously, and the animals in the three groups had differentiated remarkably by the time I first saw them. All were able to grow and maintain sufficient vitality to conceive; but the wheat-fed cows deteriorated in appearance, were small of girth, rough-haired, listless, and delivered their calves some weeks before term. Their calves were undersized and were dead when born. Early in the experiment the wheat-fed cows had become blind. The oat-fed cows were in much better condition, and although they carried their young to full term, the calves were dead or moribund, but one surviving. In marked contrast to these groups the corn-fed animals were in excellent condition. They produced vigorous calves.

My assignment was to find, if possible, the cause of the differences in the quality of the three rations. The chemical criteria accepted generally at that time indicated that they were of essentially equal value as sources of nutrients for cattle. I set to work with the enthusiasm and inexperience of youth to solve the problem.

Having cast my lot in research in animal nutrition without any knowledge of the experiences of investigators in that field, and without ever having analyzed a food by the method of the Association of Official Agricultural Chemists, my fitness for a critical appraisal of current practices and beliefs or for planning an experiment in which animals were used, might well have been assessed by an experienced man at near zero. This I realized and began at once to make good my deficiencies.

One very important source of instruction to me was my daily contact with Dr. Babcock. He was interested in everything in science, and among other things he talked about, in his visits to the laboratory where I was working, were the history of nutritional investigations, and the inadequacies of the methods used for feed and food analysis. He was a man of wisdom and my association with him almost daily was a great privilege. He did not believe that the method of analysis which had been widely accepted by experimenters in animal nutrition for over forty years yielded any information of importance concerning the nutritive values of feeds. This method had been devised by Wm. Henneberg, Director of the Agricultural Experiment Station at Weende, near Göttingen. He combined elements in the technics used by various chemists during the preceding four decades, and his method became known as the Weende method. Eventually it was modified in various details, but these were of little practical significance. It was adopted by the American Association of Official Agricultural Chemists when they first organized in 1884. It yielded information about the content of moisture, ether-soluble matter (reported as fat), "crude protein," derived by multiplying the nitrogen content by 6.25, "crude fiber" (cellulose, lignin), "nitrogen-free extract," and ash of the sample analyzed. It was assumed that the substances expressed in these terms had the same nutritive value irrespective of the plant sources from which they were derived.

After about 1860 agricultural experiment stations multiplied rapidly in several countries, and notably in the United States. Chemists analyzed enormous numbers of samples of farm crops grown under different climatic and soil conditions. These were compiled and published in the various editions of E. von Wolff's book, *The Rational Feeding of Farm Animals*. "Wolff's standards" for calculating rations for livestock were based on these analyses. His book was the basis of teaching feeding practices during four decades, wherever animal husbandry was taught. But these "standards" were often found unreliable by feeding tests, and it became increasingly evident that the methods of chemical analysis of feeds were inadequate for supplying the desired information about them. Agricultural chemists became painfully aware of their shortcomings. At the meeting of the Association of Official Agricultural Chemists in Washington in 1890 (2) the report of a committee of the foremost agricultural chemists, under the chairmanship of Dr. Harvey W. Wiley, was discussed at length. It had to do with proposed improvements in the "Official" method of feed analysis so as to provide more worthwhile data. All the suggestions offered were favorable to the determination of specific chemical constituents of the sample, especially the "nitrogen-free extract." An opinion which was supported by some was that the discrepancies between expected and realized results of feeding studies based on chemical data could be accounted for on the assumption that nutrients which were enclosed in, or imbedded in cellulose or lignin were inaccessible to digestive juices. Nothing of any importance came of these discussions, and the method then current continued to be employed for another two decades.

I read Henry's *Feeds and Feeding*, then the most popular textbook on the subject in America. From it I learned of the views of Liebig, Boussingault, Henneberg, Kellner, Rubner, Zuntz, and others who had investigated nutrition problems and feed values. I became familiar with the kinds of experiments in that field which had been employed during the preceding half-century. From these sources I learned nothing of value to me in my efforts to help Professor Hart solve the problem presented by the cow experiment. I soon came to share with Dr. Babcock his appreciation of the humor of his advice to Atwater, a story he often repeated with hearty laughter. W. A. Atwater was at that period the outstanding researcher and authority in America on human nutrition and human foods. He seems to have had no doubt that the standard food analysis, supplemented with data on calorie values and digestibility, sufficed for his purpose of determining food requirements, and for recommending economical food purchasing. Dr. Babcock told of recommending to Atwater that instead of feeding pigs on farm crops it would be cheaper to feed them soft coal. Bituminous coal, he said, when judged by the "official" method of analysis, was in itself a well-balanced ration. Dr. Atwater was annoyed by this treatment of a serious subject with levity.

The source of information which was of greatest value to me at that time was Maly's *Jahresbericht ueber die Fortschritte der Thier-Chemie*. The first volume appeared in 1872 and covered the entire literature relating to animal chemistry, and much of plant chemistry. It abstracted almost everything of importance in this field, including proteins, carbohydrates, fats and other lipids, blood, urine, digestion, pathological chemistry etc. There was a chapter under the heading *Gesamt-Stoffwechsel*, which contained abstracts of papers dealing with feeding experiments on men and animals, and their interpretation. Most of these papers described experiments designed to throw some light on nutritional needs of the body and the chemistry of nutrition. Although we had a file of this journal in the library, I bought a set of the thirty-seven volumes then published and spent many evenings at home studying their contents. It was a most profitable use of my time since these volumes made available to me the history of constructive thought and experiment in animal and plant biochemistry of the period which they covered.

It was there that I saw abstracts of the experiments of Forster (1873), Lunin (1881), Socin (1891), Hall (1896), Marcuse (1896), Steinitz (1898), Zadik (1899), Gottstein (1901), Röhmann (1902), Ehrström (1903), Falta & Nöggerath (1905), Jakob (1906), Tunnicliffe (1906), and Willcock & Hopkins (1906). These investigators restricted animals to mixtures of isolated proteins, carbohydrates, fats, and mineral salt mixtures for the purpose of comparing the nutritive values of proteins from different sources; the value of phosphorus-containing as compared with phosphorus-free proteins; the significance of feeding nucleoproteins; the effects of supplementing certain proteins with individual amino acids; or the effects of the inorganic salt content of the food on the health of the animals (3).

As I made notes on these studies I was astonished to find that every effort which had been made to feed animals on such mixtures had resulted in prompt failure of their health. It came to my mind that the most important discovery to be made in nutrition would be the elucidation of the cause or causes of these failures.

Reflection on this type of experiment as compared with the cow project with the single-plant rations—seed, leaf, and stem, which were extremely complex chemically, led me to conclude that we were not likely to succeed in accomplishing more than giving an account of what we did in this unusual study, and describing the physiological effects of the rations on the animals, but without discovering the causes, which obviously lay in chemical differences in the feeds.

It was such considerations that led me to conclude that the only promising course lay in the use of the simplest possible diets in the chemical sense, and of employing small animals, as some of the few men here recorded had done, and to make an effort to solve the problem of what, in chemical terms, constitutes the minimum quota of chemical substances on which an animal can function normally. The necessary chemical work in the preparation of the foodstuffs required precluded the use of large animals. Small animals have short periods of growth and mature early. Their periods of reproduction and suckling of the young, and their life span are such that the life history can be observed in two to three years.

It was this plan which involved the use of rats, that I presented to Dr. Babcock on a Sunday morning in November, 1907. He was highly enthusiastic about the possible achievements which might come from nutritional research by following a plan in which we would proceed from the simple to the complex rather than attempt to find why complex natural feeds in certain combinations and from certain sources failed to sustain health. With his approval and support I was able to start experimenting with rats. Mine was the first rat colony in America maintained for nutrition studies. At first I tried to use wild rats, but they were so frightened under caged conditions and were so ferocious that I soon abandoned them for albinos which I bought from a pet-stock dealer in Chicago.

From the outset I sought to find whether the failures of earlier investigators who used diets of isolated food substances might have been caused by some deficiency of an organic phosphorus compound. Professor Hart was devoting much study to this aspect of nutrition. While at the Geneva Experiment Station, in cooperation with Director W. H. Jordan and A. J. Patten, he had investigated the nutritional significance of the newly discovered organic phosphorus compound, phytic acid, and its salts "phytin," for cows. They had already published their results, which seemed to show that this substance exerted specific beneficial effects on the physiology of the cow (4). In 1909 I published the results of a considerable number of experiments bearing on this subject, in a paper *Nuclein Synthesis in the Animal Body* (5). In it I brought forward evidence for the belief that, in planning experimental diets, all known organic phosphorus compounds such as

lecithin, cephalin, nucleic acid, and phosphoprotein, which are prominent constituents of animal tissues, could be omitted, since they were all capable of synthesis in the animal body. In this paper I reviewed the work of the investigators listed above who had studied nutrition with simplified diets composed of more or less purified food substances (3).

At first I did all the work necessary for preparing foods, making rations and caring for the rats, but in the summer of 1909 Miss Marguerite Davis, who had just graduated at the University of California at Berkeley, became my student. She had not been long at work in that status when I told her what I was attempting to do with the rats, and she volunteered to take care of the colony for me. She remained with me on a voluntary basis without pay except during the sixth and last year of our work together. I continued to prepare the food materials and to plan the experiments and assist in weighing the animals in order to observe them carefully, while she otherwise had all the care of the colony. I owe her a debt of gratitude for her enthusiasm and loyalty to the undertaking. Without her co-operation it would have been impossible for me to have carried out so extensive an experimental program as we did working together.

During my early years at the College of Agriculture I wrote letters to Dr. Mendel and kept him informed on what I was trying to do, since he seemed enthusiastic about my experimental work. He commended me for undertaking studies with purified diets and seemed greatly pleased when he read my paper in 1909 (5) in which I gave my reasons for believing that all organic phosphorus compounds could be synthesized in the body. In 1909 he and Dr. Osborne started their rat colony for the study of differences in nutritive values of proteins from different sources.

From the outset of my experiments with "purified" diets I met with little success, my animals failing to grow, and showing signs of malnutrition. In seeking to overcome the failure of the rats to eat these insipid mixtures I was influenced by the work of Pavlov on the psychic reaction of animals to food and the response of the digestive glands to the chemical composition of the food ingested. I sought to overcome the difficulty of anorexia by giving as great a variety of isolated and recombined nutrients as possible, changing the source of food from time to time and providing flavor by such means as adding daily to the diet freshly rendered bacon fat, the distillate from water in which cheese was immersed, employing carbohydrates from different sources, etc., but without much success. Curiously, I did not discern the possible significance of feces-eating by my rats, and this disturbing element, together with unsuspected impurities in some of my materials, especially in milk sugar, enabled the animals to grow sufficiently to keep me enthusiastic about eventually achieving success. By reason of a combination of defects in my technic my rats were able to distinguish clearly between the value to them of butter fat and egg-yolk fat, in contrast to olive oil and lard. They fared markedly better nutritionally on the two former than on the two latter adjuvants to the diets. This study Miss Davis and I published in

1913 (6). It afforded the first evidence that certain fats contain an indispensable nutrient hitherto unsuspected. Some months later Osborne & Mendel described experiments which confirmed our discovery that certain fats contain an unidentified nutrient essential for the nutrition of the rat. Of special interest was our transfer of this nutrient from butter fat to olive oil. Butter fat was saponified in alcoholic KOH and the resulting soap was dissolved in water and olive oil was thoroughly emulsified in the soap solution. The olive oil was of the same sample as had been tested on rats and found of no value to them. The emulsion was then broken with ether, and the olive oil was recovered in that solvent. After removing the ether the olive oil was found by feeding tests to have acquired the nutritive quality of the butter fat.

In a short time we were able to demonstrate that this nutrient, now known as vitamin A, was present in kidney fat and in fats from other glandular organs and also in the ether extract of the leaves of plants, but was absent, generally, from the fats of adipose tissues.

Following up the idea of observing the effects of diets of the simplest possible composition, I restricted young rats to single kinds of seeds: maize, wheat, oats, barley, rye, peas, beans, millet, etc. To my surprise I found that young rats restricted to any one of these were able to grow but little or not at all. Even combinations of two, three, or more seeds in this list, as the sole diet, did not support growth. This type of ration was, of course, much simpler, chemically, than the rations derived from all parts of the plant, which had been fed the cows; it was also more satisfactory for critical study.

I had devoted considerable attention to the published analyses of the ash constituents of various food substances and was impressed by the fact that the seeds of plants were all low in their calcium content. They differed considerably depending on the type of soil on which the plants had been grown. The great activity in the study of the chemistry of proteins revealed their pronounced differences in yields of amino acids on hydrolysis, and this suggested that the deficiencies of seeds might lie, solely or partly, in the peculiarity and inferiority of their proteins. The absence of the fat-soluble factor from vegetable oils and fats obtained from parts other than leaves, afforded another clue to the planning of experiments with rats to reveal the nature and number of nutrients in which seeds were deficient.

Our first experiments of this type were conducted with wheat (7). The results were as follows: (*a*) Wheat alone: no growth, short life; (*b*) wheat +purified protein (casein): no growth, short life; (*c*) Wheat+salt mixture to give it a mineral content similar to that of milk: very little growth; (*d*) Wheat+a "growth-promoting" fat (butter fat): no growth; (*e*) Wheat +protein+salt mixture: good growth for a time, few or no young, short life; (*f*) Wheat+protein+butter fat: no growth, short life; (*g*) Wheat+salt mixture+butter fat: fair growth for a time, few young, short life; (*h*) Wheat+protein+the salt mixture+the "growth-promoting" fat: good

growth, normal number of young, low mortality among the young, and long life span. Other experiments showed that by far the most important constituent in the salt mixture for making good the inorganic deficiencies of wheat was calcium.

On testing the maize kernel and the seeds of barley, rye, and millet, we found that as food for young rats, each had the same deficiencies as wheat, each requiring the same supplements of protein, salt mixture and "growth-promoting" fat. It was now clear that all of our common cereal grains are deficient in the same nutrients and approximately to the same extent. Oats are an exception since with the three additions named the response was less satisfactory than with the other three grains. Years later this was shown by others to be due to the low riboflavin content of oats.

These observations accounted for our failure to secure appreciably better results with young rats fed combinations of two or more cereals. Our experiments demonstrated that, nutritionally, all seeds have the same shortcomings. Hence when used in combination they do not make good each other's deficiencies.

Whole rice proved to be much like wheat in its dietary properties, but polished rice was not made complete nutritionally by the three types of nutrients which made wheat, maize etc. complete. But when a fourth adjuvant in the form of three per cent of wheat germ, or the water or alcohol extract equivalent to three to five per cent of wheat germ, was added, polished rice became complete nutritionally. Extracts of various foods made with either water or alcohol also provided the necessary nutrients to render rice plus the three supplements complete (8).

By the time we had reached the study of rice I was familiar with the contents of Funk's book *Die Vitamine* (9). Among other information new to me it contained an account of the effects of water, or alcohol, extracts of rice polishings on polyneuritic birds, and the value of rice polishings as a supplement to polished rice in nutrition.

One of our most interesting findings of those years was the high nutritional value of mixtures of seed with leaf as against the slight improvement of the dietary value of mixtures of seeds of plants (10). Our studies made it clear that irrespective of what chemical analysis might show, the seed is inferior to the leaf as a source of nutrients. This was in harmony with the observation which is as old as history, that animals flourish when confined to good pasture grasses and to good hay.

My observations of the effects of such diets as those just described afforded the basis for reflection on the quality of human dietaries in different parts of the world—the coldest, the wettest, and the dryest regions. The new knowledge of the dietary properties of seed, leaf, milk (which we found to be an excellent supplement to seeds), and some observations of the dietary deficiencies of muscle meat, together with the new information about polished rice and the superiority of the germ as a source of nutrients, led me to make some important generalizations on human dietaries. I criticized the typical

American's diet of that period as being of poor quality because it was derived too largely from white flour or cornmeal, muscle meats, potatoes, and sugar. Sugar, I asserted, when eaten to the extent of an average of more than 100 pounds per capita per annum, crowded out from the diet significant amounts of better constituted foods. The foods listed, I declared, were not constituted to supplement each other by making good their deficiencies. I recommended a diet containing more milk and leafy vegetables, and extolled the glandular organs of animals as superior to the muscle meats as sources of nutrients. Milk and leafy vegetables I distinguished as "protective foods" because they were so constituted as to make good the deficiencies of whatever else we were likely to eat. The planning of menus to include sufficient of these "protective" foods was recommended in my Harvey Lecture of 1917 (11), and in my Cutter Lectures at Harvard University in 1918, which were published as the first edition of *The Newer Knowledge of Nutrition* (12).

Recent practices in menu-planning stem from the principle of making the daily menus dietetically complete by the use of foods and food combinations which supplement each other. This viewpoint superseded that of Atwater which was based on the economic principle of the purchasing of those foods which would provide at lowest cost the necessary amounts of protein and available calories to meet the individual's needs.

Even in 1911, after four years of experience with feeding "purified" diets, I was still deluding myself with the idea that such success as I had achieved was the result of inducing my rats to eat enough of unpalatable mixtures to enable them to grow to some extent, and that this was the only impediment to further success in this type of study. I was awakened to my error in 1911 when Osborne & Mendel published the results of the first two years of study of nutritional differences in the values of proteins from different sources (13). Dr. Osborne had accumulated a superb collection of many highly purified proteins from vegetable sources, and in 1909 he and Mendel had undertaken to evaluate these by feeding studies in which they employed diets containing but a single protein. Their early efforts were based on diets derived from a purified protein, a source of carbohydrate, fat, and a salt mixture. They had the same experience as their predecessors in securing but minimal amounts of growth in their young rats. Metabolism studies on individual rats showed that positive nitrogen balances could be achieved over a period of three weeks, but their animals failed nutritionally before many weeks, and rapid and sustained growth was never observed. It became evident to Osborne & Mendel that some other type of basal diet must be employed for the realization of their objective.

They then turned to the use of a basal diet consisting of 28 per cent of a deproteinated whey made by coagulating the lactalbumin from acidified whey by heat, and evaporating the filtrate to dryness. This material they termed "protein-free milk." It was a mixture of the salts of milk, lactose, and of the numerous non-protein constituents of milk. When they employed

this material together with starch and lard, and certain individual proteins they were successful in inducing growth and maintaining health, and in some cases reproduction, in rats. Certain proteins, fed in this manner, were inadequate, but were made adequate by a supplement of one or more amino acids. The extraordinary differences in nutritive values of proteins from different sources were first dramatized by them.

At the time it seemed to me that "protein-free milk" was more than a source of dietary adjuvants other than protein. I pointed out that when they fed 18 per cent of a purified protein with 28 per cent of "protein-free milk," the latter supplied 9 per cent of the total nitrogen of the diet in uncharacterized substances, some of which were presumably amino acids, peptides etc. which could supplement amino acid deficiencies in purified proteins. Hence what they were accomplishing was a comparison of a purified protein plus the amino acid supplement in the "protein-free milk," with another purified protein with the same supplement. This, of course, did not detract from their demonstration of the fact that proteins differ enormously in their adequacy as sources of amino acids, a fact which was in harmony with much chemical data, especially those which Dr. Osborne had published.

Their success in improving diets by the inclusion of the non-protein constituents of milk, led me to re-examine my milk sugar as a possible source of nutrients other than lactose. It at once emerged that lactose purified by re-crystallization was less valuable to rats restricted to my experimental diets than was the crude sugar, and that addition of the mother liquor from crystallization of milk sugar had an easily observable beneficial effect on the animals.

In 1913 Osborne & Mendel reported their experiences with an "artificial protein-free milk," prepared from milk sugar and the salts of milk. With this they achieved considerable success for a few weeks but the rats failed in health within about one hundred days, whereas with the natural "protein-free milk," they remained in good health far beyond this age.

Osborne & Mendel (13) rendered a service to animal experimenters in the field of nutrition by calling attention to the beneficial effects to rats fed "purified" diets, of feces-eating, a practice to which this species is addicted. They observed that a small allowance of feces, more especially from animals normally fed, was of considerable value in improving their well-being. They were led to try feeding feces by the recently reported studies of Herter & Kendall (14) which afforded strong evidence that certain types of bacterial flora in the intestines are physiologically beneficial, whereas others are harmful.

The investigations here described represent the principal ones which I carried out in the decade under consideration, which embodied new and novel features. They were well received by biochemists and physiologists. The comment on my work in 1917 by Professor Graham Lusk gave me

great pleasure. He said to me: "You have entered a well-worked field and have brought forth new and astonishing facts." My investigations increased my visibility as a researcher sufficiently to prompt Dr. Wm. H. Welch and Dr. Wm. H. Howell to invite me to take charge of the department of chemistry in the newly established School of Hygiene and Public Health which the Rockefeller Foundation had made possible as a part of the Johns Hopkins University. I have never ceased to marvel that these two distinguished medical men should have risked appointing me to a professorship when I had no medical training and was a chemist working in an agricultural experiment station.

Since this is intended to be an historical account of my first decade in research, it seems desirable that I should mention the more important investigations previously and currently, which, in addition to those mentioned, contributed to an understanding of the problem of what constitutes an adequate diet.

Lunin (3) was inspired by his teacher, v. Bunge, to study the physiological importance of inorganic elements in nutrition. His specific problem was to find whether it was important to take into account the acid-base balance in foods. To this end he attempted to keep mice on a diet composed of what he believed to be the essential ingredients in milk, viz., casein, milk sugar, fats, and the ash of milk. His mice died within a few weeks on this mixture, whereas when given milk to drink they remained in health for at least sixty days. He wrote:

> Mice can live well under these conditions when receiving suitable foods (e.g. milk), but as the experiments show that they cannot subsist on proteins, fats and carbohydrates, salts and water, it follows that other substances indispensable for nutrition must be present in milk besides casein, fat, lactose and salts.

He contributed nothing further to this subject.

Pekelharing, in 1905, restricted mice to a diet of bread made of casein, albumen, rice flour, lard, and a mixture of all the salts which he thought should be present in their food. When they were given this ration with water they grew thin and died within four weeks. When they were given milk in addition to the bread they remained in health. He further showed that a whey allowance with the experimental diet would keep the mice healthy. He wrote:

> My purpose is to point out that there is a still unknown substance in milk, which, even in very small quantities, is of paramount importance to nourishment. If this substance is absent, the organism loses the power properly to assimilate the well-known principal parts of the food, the appetite fails, and with apparent abundance the animals die of want.

I did not learn of the study of Pekelharing until it was brought to my attention about 1923 by his countryman Dr. van Leersum. It was not recorded in Maly's Jahresbericht.

In 1906 F. G. Hopkins wrote (15):

But further, no animal can live upon a mixture of proteins, carbohydrates and fats, and even when the necessary inorganic material is carefully supplied, the animal still cannot flourish. The animal body is adjusted to live either on plant tissues or on other animals, and these contain countless substances other than proteins, carbohydrates and fats. Physiological evolution, I believe, has made some of these well nigh as essential as are the basal constituents of the diet . . . ."

The studies of Grijns (19) corrected the initial error of Eijkman (18) in interpreting his famous experiment on the production of polyneuritis in fowls by restricting them to a diet of polished rice. Eijkman proposed to explain the observed phenomena on the presence in the endosperm of rice of a nerve poison for which there was in the outer layers of whole rice a substance which neutralized this in the pharmacological sense. Grijns (19) was the first to interpret correctly the connection between excessive consumption of polished rice and the etiology of beri-beri.

In 1902 Holst & Froelich (16) made the momentous discovery that any diet which was thoroughly dry or thoroughly heated would induce scurvy in guinea-pigs, whereas fresh, unheated vegetable foods would prevent or cure it. Hitherto, views about the cause and cure of scurvy had been based on human experience, and well-controlled studies were out of the question. But with the guinea-pig as a subject for experimental scurvy, progress was to be rapid in securing sound knowledge to replace the divergent views which had hitherto prevailed as to the etiology of the disease. At this period boiled milk and barley water formulas were commonly fed to artificially reared infants, and infantile scurvy was common. On the suggestion of Holst & Froelich's experiments A. F. Hess in 1914 (17) substituted potato water for barley water and promptly cured scurvy in an infant. Dr. Hess was very active in educational work which resulted in the practically universal provision of some fresh fruit or vegetable juice to bottle-fed infants. The incidence of infantile scurvy was markedly reduced.

The greatest impediment to progress in nutrition studies up to 1917, or even somewhat later, was the biochemists' lack of training in pathology and the pathologists' lack of training in chemistry. Knowledge of the meaning of symptoms exhibited by experimental animals in states of malnutrition due to confinement to diets from different sources, and deficient in different nutrients, would have shed much light on the interpretation of feeding studies which caused specific kinds of malnutrition. I keenly realized my deficiency in this respect and sought assistance from medically trained men and from professional pathologists and veterinarians. None of these men manifested much interest in the meaning of the photographs of experimental rats, cows, calves, pigs, and chickens which I carried about. To questions about the meaning of abnormal posture, skin and eye lesions, etc. I received no helpful replies. The pathologists of that time were informed in morbid anatomy, the natural history of disease, in the roles which bacteria, fungi

and the protozoa played as agents in the causation of disease, and in immunology. None had sufficient training in chemistry to enable him to conceive of a diseased state arising from a deficiency of some essential chemical substance which the diet must supply. Disease at that time was generally regarded as due to some positive agent.

Practical feeders had long known that certain feeds were better than others as supplements to some farm crop, e.g. hay or silage. Feed-lot tests at many experiment stations had revealed various combinations of feeds which produced better results than other combinations apparently similar. The reasons remained unknown until the decade here discussed.

From the account here given of prior investigations it will be apparent that feeding studies on laboratory and farm animals afforded a number of lessons of importance which up to 1907 had not been studied with much profit. Lunin's experiments were twenty-eight years old and nothing had been done to advance knowledge beyond the facts which he recorded. The time was ripe for more systematic experimental inquiry, prosecuted to an extent which would confirm, clarify, extend, and unify the isolated observations of importance which were known to practical feeders, or were recorded in scientific journals. Some of these were of importance, but had not been followed up as they should have been by further study. I was fortunate to have opportunity and resources for extensive experimental studies in animal nutrition at a most opportune time.

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BIOLOGICAL OXIDATIONS[1,2]

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In this review, emphasis has been placed on the enzymic mechanisms of the oxidation of intermediary metabolites, that is on the reactions involving the hydrogen atoms (or electrons) of the substrate rather than the carbon atoms. The sequence of reactions of the carbon skeleton of these compounds, such as described in the Krebs tricarboxylic acid cycle or in the "oxidative pathway" of glucose-6-phosphate metabolism, will doubtless be considered in another chapter of this volume. Fatty acid oxidation has been omitted, and fields in which only a few papers have appeared, e.g. the copper-containing oxidases, are also not considered.

Nomenclature.—A uniform nomenclature of oxidizing enzymes will be employed. An enzyme will be called an oxidase only when it reacts directly with molecular oxygen; if more than one enzyme is involved, the term "oxidase system" will be employed. "Dehydrogenase" will be used to refer to the enzyme which reacts directly with the substrate. These terms will be used only to describe enzymes catalyzing a one-step oxidation (two hydrogen atoms or electrons, except in the case of cytochrome oxidase, which involves only one electron). For example, "pyruvic oxidase system" means the system of enzymes required for the oxidation of pyruvate by oxygen to the level of acetate, not for the complete oxidation of pyruvate to CO_2 and H_2O.

Pyruvic and α-Ketoglutaric Oxidase Systems

Much progress has been made during the last year in the laboratories of Green, Gunsalus, Lipmann, and Ochoa towards the elucidation of these complicated systems. Reviews have been published by Barker (2), Green (3), Ochoa (4, 4a), and Lipmann *et al.* (5).

Pyruvic oxidase system.—The occurrence of reaction 1 [R·SH = coenzyme A (CoA); DPN^+ = diphosphopyridine nucleotide] in extracts of heart and

[1] This review covers approximately the period October 1951 to September 1952. Many papers which appeared within this period have been covered by the previous reviews (1, 4a) and have been omitted.

[2] The following abbreviations are used: ADP for adenosinediphosphate; AMC for acetylmethylcarbinol; AMP for adenosinemonophosphate; ATP for adenosinetriphosphate; BAL for 2,3-dimercaptopropanol (anti-Lewisite); CoA for coenzyme A; DHA for dehydroascorbic acid; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); DPT for diphosphothiamine; FAD for flavin-adenine-dinucleotide; GSH for glutathione (reduced form); GSSG for glutathione (oxidized form); HMP for hexosemonophosphate; TPN for triphosphopyridine nucleotide; TPNH for triphosphopyridine nucleotide (reduced form).

pigeon breast muscle and microorganisms has now been firmly established by work in three laboratories.

$$CH_3 \cdot CO \cdot COOH + R \cdot SH + DPN^+ \rightarrow CH_3 \cdot CO \sim SR + DPNH + CO_2 + H^+ \quad 1.$$

Korkes *et al.* (6) first obtained indirect evidence for this reaction in bacterial and heart muscle extracts, which required DPN[2] and CoA[2] as catalysts for the dismutation of pyruvate; the DPNH was oxidized by another molecule of pyruvate (in the presence of lactic dehydrogenase), while CoA was regenerated from the acetyl derivative by either reaction 2 or 3:

$$CH_3 \cdot CO \sim SR + H_3PO_4 \rightleftharpoons CH_3 \cdot CO \cdot O \sim H_2PO_3 + R \cdot SH \text{ (phosphotransacetylase)} \quad 2.$$

$$CH_3 \cdot CO \sim SR + \text{oxalacetate} \rightleftharpoons \text{citrate} + R \cdot SH \text{ ("condensing enzyme")} \quad 3.$$

Phosphotransacetylase, which is found only in bacterial extracts, and the more widely distributed "condensing enzyme" have been purified by Stadtman (7) and Stern & Ochoa (8) respectively. Other "acetokinase" (8a) reactions of acetyl-CoA leading to the regeneration of the free coenzyme have been reviewed by Barker (2) and Lipmann *et al.* (5, 8a). One recently discovered is reaction 4, catalyzed by acetyl-CoA deacylase (6) (10).

$$CH_3 \cdot CO \sim SR + H_2O \rightarrow CH_3 \cdot CO \cdot OH + R \cdot SH \quad 4.$$

Jagannathan & Schweet (11), using enzyme preparations first from pigeon breast muscle and later from heart muscle, obtained similar indirect evidence for reaction 1. The formation of acetyl-CoA was, in this case, suggested by the formation of acetyl-sulphanilamide after the addition of sulphanilamide and a liver enzyme.

The formation of acetyl-CoA has now been definitely proved. Korkes [see Ochoa (4)] has isolated acetyl-CoA after dismutation of pyruvate by extracts of *Escherichia coli* in the absence of acetyl-acceptor systems. Littlefield & Sanadi (11b) have also isolated acetyl-CoA from the products of the oxidation of pyruvate by DPN, using the purified enzyme (11). Reaction 3 has been shown directly (8) by using crystalline "condensing enzyme" and acetyl-CoA isolated from yeast by Lynen & Reichert (9). The reduction of DPN[2] has been demonstrated directly by Gergely *et al.* (10) with purified pyruvic dehydrogenase, using phosphotransacetylase and arsenate to react with the acetyl-CoA.

Korkes *et al.* (6) separated the protein fraction required for reaction 1 in *E. coli* into two enzyme components (A and B) and comparable fractions from *Streptococcus faecalis* have been obtained by Gunsalus (12). The heart muscle enzyme system has not been resolved in this way, although a protein fraction able to replace enzyme B in the *E. coli* system can be prepared from heart. The claim of Schweet *et al.* (11) to have separated the heart enzyme system into two components now seems doubtful, since the second component was probably deacylase required not for the oxidation step but for regeneration of the CoA (cf. 10). Ochoa (4, 4a) has suggested that reaction 1 is the sum of the following:

$$\text{Pyruvate} + \text{X} \rightleftharpoons \text{acetaldehyde—X} + CO_2 \qquad 5.$$

$$\text{Acetaldehyde-X} + \text{CoA} \rightleftharpoons \text{acetaldehyde-CoA} + \text{X} \qquad 6.$$

$$\text{Acetaldehyde-CoA} + \text{DPN}^+ \rightleftharpoons \text{acetyl-CoA} + \text{DPNH} + H^+ \qquad 7.$$

Since Korkes has found that C^{14} is incorporated in the carboxyl group of pyruvate when enzyme A, $C^{14}O_2$, pyruvate, and diphosphothiamine (DPT) are incubated in the absence of CoA [see also Goldberg & Sanadi (11a)], Ochoa suggests that X is DPT and that enzyme A catalyzes reaction 5 [cf. Singer (15)]. He suggests that enzyme B catalyzes reaction 7, since it can be replaced by *Clostridium kluyveri* extracts which oxidize acetaldehyde but not pyruvate [see also Burton (12a)]. However, Reed & DeBusk (17) assign a different role to enzyme B (see p. 20).

An essential feature of Ochoa's mechanism is that CoA enters the reaction before the dehydrogenation step. Jagannathan & Schweet (11) found, however, that the preparation from pigeon breast muscle (but not the heart preparation) is able to utilize for the oxidation of pyruvate to acetate a number of artificial hydrogen acceptors (e.g., ferricyanide or dyestuffs) in a reaction not requiring CoA, DPN, or inorganic phosphate; DPT and Mg^{++} were the only requirements for full activity. This suggests that the dehydrogenation step might precede the transfer of acetyl.

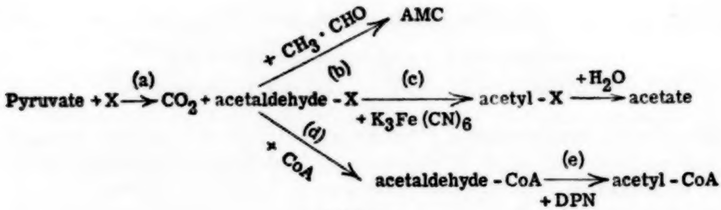
Many preparations which decarboxylate pyruvate, whether oxidatively or nonoxidatively, are able to catalyze the formation of acetylmethylcarbinol (AMC) from pyruvate (reaction 8) or acetaldehyde.

$$2\,CH_3 \cdot CO \cdot COOH \rightarrow CH_3 \cdot CO \cdot CHOH \cdot CH_3 + 2\,CO_2 \qquad 8.$$

Jagannathan & Schweet's pigeon breast muscle preparation, but not that from heart, has AMC-forming activity, and this activity remained proportional to the reduction of ferricyanide throughout purification and various inactivating treatments. Since the AMC-reaction is, in its overall result, a dismutation of acetaldehyde, it has been actively studied in the hope that some light will be thrown on the dehydrogenation step. Juni (13) has studied the differences between the reaction in bacteria, yeast, and animal cells; acetolactate is an intermediate only in bacteria. Singer & Pensky (15) have found that highly purified wheat germ α-carboxylase forms AMC[2], and they propose a unifying theory by which the differences between the enzymes from different sources are explained in terms of their respective dissociation constants for acetaldehyde and other aldehydes. Dolin & Gunsalus (14) have found that the AMC-reaction is hardly detectable in resting cell suspensions of *S. faecalis* which oxidize pyruvate to acetate, but is the predominant reaction in cell-free extracts.

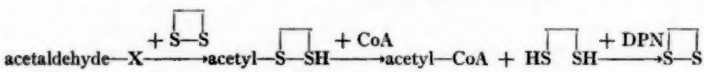
Scheme A reconciles Ochoa's mechanism with Jagannathan & Schweet's findings. Reaction (c) may be catalyzed by what can be termed "C_2-dehydrogenase," or it may be entirely artificial.

Gunsalus (16a) proposes that the dehydrogenation step precedes the



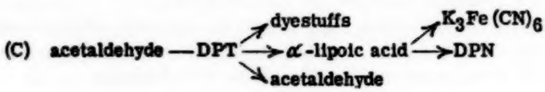
Scheme A

transfer of acetyl to CoA[2] and introduces α-lipoic acid into the reaction mechanism. He would replace reactions (d) and (e) by Scheme B.



Scheme B

X, as in Ochoa's scheme, is DPT[2] and S—S is the cyclic disulphide, α-lipoic acid. The hyrdogen-tranfer in Gunsalus' scheme may be written as Scheme C:—



Scheme C

where the arrows now represent the pathway of the hydrogen atoms. Gunsalus' scheme is more analogous to other multicomponent hydrogen transferring systems since the component which reacts with an artificial hydrogen acceptor also reacts with the naturally occurring hydrogen carrier. This scheme is consistent with Dolin & Gunsalus' (14) finding that α-lipoic acid is necessary for the oxidation of pyruvate, but not for the AMC-reaction, and with the requirement of lipoic acid for acetylation by extracts of *Tetrahymena geleii S.* but not for oxidation of pyruvate by dyestuffs [Seaman (18)]. However, α-lipoic acid is necessary for the reaction with ferricyanide (16a).

Reed & DeBusk (17) find that an extract of a mutant of *E. coli* requires the amide of α-lipoic acid and DPT (called lipothiamide pyrophosphate) for the oxidation of pyruvate and α-ketoglutarate (DPT plus α-lipoic acid is inactive). They believe that this mutant lacks Ochoa's enzyme B, whose function in the wild type is to form lipothiamide pyrophosphate from DPT and bound α-lipoic acid.

The system in *Proteus vulgaris* extract is of particular interest. Moyed & O'Kane (16) have shown that pyruvate is aerobically oxidized to acetate and CO_2 by these extracts without the participation of CoA or α-lipoic acid.

The extracts have been separated into two fractions: (*a*) a soluble fraction (purified 60-fold) which is sufficient, with added DPT, for the reduction of dyestuffs by pyruvate; (*b*) a particulate fraction which contains cytochromes-b_1, a_1, and a_2. The aerobic oxidation of pyruvate requires the two fractions, plus DPT. In terms of scheme A, it seems that the soluble fraction catalyzes reaction (a)+(c), while the cytochrome system in the particulate fraction can replace $K_3Fe(CN)_6$ in reaction (c). Thus, in this organism at least, reaction (c) can be carried out with the naturally occurring hydrogen-carrier. It is particularly interesting, moreover, that the pathway (d), (e) of scheme A or scheme B can be brought into play by the addition of CoA[2] and DPN to these extracts. Despite the many different types of organisms used by investigators in this field, the same general mechanism appears to fit all the results; the differences seem to be in the relative rates of the different reactions of acetaldehyde-X.

Lynen (9) and Ochoa (4a, 6) postulate that the actual dehydrogenation in the pyruvic oxidase system occurs while the carbonyl group of pyruvate or the acetaldehyde derivative is combined to an —SH group, probably that contained in CoA. Ochoa & Stern (4a) support this view with the experiments of Cavallini (20) on the coupled oxidation of pyruvate and glutathione (GSH) in the presence of Cu^{++} ions as a chemical model of the enzyme system. Cavallini postulated the formation of a GSH-pyruvate addition product which was subsequently oxidized. Slater (21) has shown, however, that the oxidation of pyruvate is due, not to compound formation, but to the oxidation of pyruvate by H_2O_2 formed in the oxidation of GSH.[2]

$$2\ GSH + O_2 \rightarrow GSSG + H_2O_2 \qquad 9.$$

$$CH_3 \cdot CO \cdot COOH + H_2O_2 \rightarrow CH_3 \cdot COOH + CO_2 + H_2O \qquad 10.$$

He found that the oxidation of pyruvate could be prevented by the addition of catalase, so that Cavallini's system cannot be taken as a model of the enzyme reaction. This does not necessarily disprove the idea that pyruvate might combine with —SH groups in the enzyme system; Racker (22) has evidence that methylglyoxal combines with GSH in the glyoxalase system (see also p. 41).

It is clear that reaction 1 includes a number of steps, all of which appear to be carried out by the one fairly homogeneous enzyme preparation, with a mean molecular weight of 4×10^6 (11). The isolation of such a protein fraction [and the analogous α-ketoglutaric dehydrogenase preparation (26)] which can be easily separated from, on the one hand, the larger particles which comprise the terminal respiratory chain and, on the other, smaller protein molecules such as acetyl-CoA deacylase or Ochoa's condensing enzyme is a most important development. Although Green (3) considers that reaction 1 is catalyzed by a single protein with five prosthetic groups (α-lipoic acid, CoA, DPN, DPT, and Mg), it would not be very surprising if the complex of high molecular weight were eventually split into proteins of smaller molecular weight, each possessing perhaps one or two prosthetic

groups. Some progress in this direction has been achieved in the laboratories of Ochoa & Gunsalus. It would also not be surprising if problems of mutual accessibilities of the individual components of the system, met with in studies of the larger respiratory particles [Keilin & Hartree (19)], are found with these complexes of intermediate molecular weight.

α-Ketoglutaric oxidase system.—Kaufman (23) in Ochoa's laboratory showed that CoA as well as DPN was required for the dismutation of α-ketoglutarate, catalyzed by a soluble heart muscle preparation. He suggested that DPN[2] was reduced according to equation 11, which is exactly analogous to equation 1.

$$\alpha\text{-ketoglutarate} + DPN^+ + CoA \rightarrow \text{succinyl-CoA} + DPNH + H^+ + CO_2 \qquad 11.$$

In the dismutation, the DPNH is re-oxidized by the glutamic dehydrogenase reaction:

$$DPNH + \alpha\text{-ketoglutarate} + NH_4^+ \rightarrow \text{glutamate} + DPN^+. \qquad 12,$$

The suggested formation of succinyl-CoA was supported by Sanadi & Littlefield (26). They coupled the oxidation of α-ketoglutarate catalyzed by pigeon breast muscle and pig heart preparations with the synthesis of succinylsulphanilamide in the presence of CoA and a pigeon liver enzyme. The succinylsulphanilamide was isolated and characterized by the usual procedures of organic chemistry. More recently these workers have isolated succinyl-CoA, using a purified pig heart preparation. This reacted with sulphanilamide in the presence of the appropriate enzyme and also nonenzymically with hydroxamic acid.

Simultaneously with Kaufman's work, Gergely & Hele (24) in Green's laboratory showed the direct reduction of DPN[2] by α-ketoglutarate, in the presence of a soluble enzyme and an unknown coenzyme later identified as CoA. This direct reduction of DPN in the presence of CoA was confirmed by Kaufman *et al.* (25). Gergely *et al.* (10) and Sanadi & Littlefield (26) have shown that when DPN is in excess the amount of DPNH formed corresponded to the amount of CoA. If at this point succinyl-CoA-deacylase was added, the DPN became completely reduced.

Since succinylation is not so common a reaction as acetylation, there are fewer reactions involving succinyl-CoA than acetyl-CoA. Known reactions are:

$$\text{Succinyl-CoA} + H_2O \rightarrow \text{succinate} + CoA\ (23, 10) \qquad 13.$$

$$\text{Succinyl-CoA} + ADP + H_3PO_4 \rightleftharpoons \text{succinate} + CoA + ATP\ (23, 27) \qquad 14.$$

$$\text{Succinyl-CoA} + \text{acetoacetate} \rightarrow \text{acetoacetyl-CoA} + \text{succinate}\ (3) \qquad 15.$$

Succinyl-CoA may also be involved in the biosynthesis of porphyrin [Shemin (28)]. There may be other exchange reactions like reaction 15. Reaction 14 is of particular interest since it generates energy-rich phosphate from inorganic phosphate, first shown to be coupled with the dismutation of α-ketoglutarate by Hunter & Hixon (29). Kaufman (23) and later Littlefield & Sanadi (27) showed that this phosphorylation was catalyzed by the soluble

preparations and suggested equation 14. The yield of ATP was less than theoretical owing to the presence of deacylase (reaction 13). There is as yet no evidence that reaction 14 has its counterpart in the pyruvic oxidase system of animal tissues where the energy of the acyl-CoA derivative is probably largely utilized in the synthesis of citrate, not the generation of energy-rich phosphate bonds. Kaufman found that inorganic phosphate was liberated from ATP in the presence of succinate, CoA, and the enzyme, showing that reaction 14 was reversible. This reaction is further discussed on p. 33.

The mechanism of hydrogen transfer in the α-ketoglutaric oxidase system is probably very similar to that in the pyruvic oxidase system. α-Lipoic acid is certainly involved in *E. coli* (17), while Sanadi's purified preparation contains 6 molecules of α-lipoic acid per molecule of protein (26). This preparation which has been purified 350-fold is nearly homogeneous in physical behavior with a molecular weight of 2×10^6. Like the analogous pyruvic dehydrogenase (11), it does not require DPN or CoA for reduction of artificial hydrogen acceptors. The α-ketoglutaric dehydrogenase preparation also catalyzes the decarboxylation of α-ketoglutarate to succinic semialdehyde as well as an unidentified nonoxidative side reaction. Holton & Slater (30) have found that some unknown heat-stable substance in crude yeast hexokinase preparations promotes the anaerobic disappearance of α-ketoglutarate when added to heart muscle sarcosomes (mitochondria). The reaction products have not yet been identified. This anaerobic reaction is accompanied by phosphorylation.

In experiments *in vitro*, DPNH[2] formed by reactions 1 or 11 has been reoxidized by pyruvate and lactic dehydrogenase or α-ketoglutarate, ammonia, and glutamic dehydrogenase respectively. If DPN is in fact the acceptor of the hydrogen atoms of pyruvate and α-ketoglutarate in the cell, the DPNH is more likely oxidized by the DPNH oxidase system, which has been removed from the extracts. It is not yet certain that DPN is the hydrogen acceptor in the cell, since it is possible that in an intact system an intermediate carrier in reactions 1 or 11 might react directly with a flavoprotein or with the cytochrome system without the mediation of DPN. A situation analogous to this possibility is the reduction under anaerobic conditions of cytochrome-*b* by DPNH, although cytochrome-*b* is not a component of the DPNH oxidase system. This has previously been explained by scheme D [Slater (31)]

$$\begin{array}{c} \text{Cytochrome-}b \\ \upharpoonleft\!\downharpoonright \\ \text{DPNH}\rightarrow\text{Diaphorase}\rightarrow\text{Intermediary factor}\rightarrow\text{Cytochrome-}c \end{array}$$

Scheme D

where the arrows show the path of hydrogen or electron transfer.

Respiratory Chain

By respiratory chain is meant the system of enzymes which transfer hy-

drogen atoms or electrons from reduced pyridine nucleotide or succinate to molecular oxygen.

Succinic oxidase system.—Tsou (32) has found that succinic dehydrogenase in particulate preparations is irreversibly inactivated by incubation with cyanide. The enzyme is completely protected from cyanide by the addition of succinate or dithionite but not by malonate or other competitive inhibitors of the enzyme, showing that it is the oxidized form of the enzyme which combines with cyanide. After cyanide treatment, cytochrome-*b* in the preparation is only very slowly reduced by succinate but it is eventually completely reduced showing that cytochrome-*b* and the dehydrogenase are not identical. Chance's (1, 33) kinetic measurements are in agreement with this conclusion. The scheme previously proposed [Slater (34)] for the succinic oxidase system should, then, be expanded as in Scheme E.

Succ.→dehydrog.→cyt.-*b*→factor→cyt.-*c*→cyt.-*a*→cyt.-a_3→O_2

Scheme E

Chance (33), by means of ingenious and sensitive methods, has measured the concentrations and rates of reduction of the cytochromes in heart muscle preparations. The concentration of cytochrome-*c*, calculated from Chance's data (0.7 μmoles cytochrome-*c* per gm. fat-free dry weight), is almost identical with the value determined by visual spectroscopy (34). Chance's determinations of the rates of reduction of cytochromes-*c*, *a*, and a_3, either during exhaustion of oxygen or in the presence of cyanide, are in excellent agreement with the participation of cytochromes-*c*, *a*, and a_3 in the system as shown in scheme E. However, on the basis of the slow reduction of cytochrome-*b* in the presence of cyanide, Chance has proposed a reaction scheme for the succinic oxidase system which is similar to that given in scheme D for the DPNH[2] oxidase system, i.e., cytochrome-*b* is in equilibrium with the dehydrogenase or the factor but is not in the main reaction chain. Although it explains the behaviour in the presence of cyanide, Chance's modification does not explain the properties of the uninhibited system. Contrary to Chance's opinion, the reviewer considers that the course of the reduction of cytochrome-*b* when oxygen is exhausted from the solution by reaction with succinate is close to what would be expected if cytochrome-*b* were in the main pathway. Chance's mathematical treatment has ignored possible back reactions, yet from Ball's (35) data, the back reaction of equation 16 must be considerable under Chance's conditions, where the fumarate-succinate ratio is 0.15 at the end of the experiment.

$$\text{succinate} + 2\,b\cdots \rightleftharpoons \text{fumarate} + 2\,b\cdot\cdot + 2\,H^+$$

$$b\cdots,\ b\cdot\cdot = \text{ferri- and ferrocytochrome-}b\text{ respectively)} \qquad 16.$$

Indeed, Ball's data suggest that only 37 per cent of the cytochrome-*b* would be reduced at the end of Chance's experiments. It is clear from Chance's data that the reduction has proceeded much further than this so that Ball's value for the potential of cytochrome-*b* may have to be raised. but in any

case the back reaction certainly cannot be ignored in any analysis of the mechanism. Chance's finding that only 68 per cent of the cytochrome-*b* is in the oxidized form in the steady state is not consistent with his mechanism.

An important extension of Chance's work is in the study of intact cells of microorganisms. This has enabled him to follow the reduction and the oxidation of pyridine nucleotides during respiration, which is the first direct evidence in the intact cell of the participation of these catalysts in respiration. He has identified reduced pyridine nucleotide both free (absorption maximum, 340 mμ) and bound (maximum at 325 mμ—see p. 50) in microorganisms. The former becomes partially oxidized upon the initiation of anaerobic glycolysis; the bound pyridine nucleotide is not oxidized, possibly because its function is directly associated with the cytochrome system. Chance & Castor (33) have shown that contrary to earlier studies, tumour cells are rich in cytochrome-*c;* they contain little cytochrome-*b*.

Slater & Bonner (36) have found that fluoride and phosphate, especially together, are competitive inhibitors of succinic dehydrogenase and have measured the inhibition constants. By studying the degree of inhibition under conditions in which the rate of the overall reaction was varied by altering the concentration of hydrogen acceptor (methylene blue or oxygen) it was possible to measure the rate constants of the reaction $E+S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES$ where E, S, and ES are succinic dehydrogenase, succinate, and the Michaelis enzyme-substrate compound respectively. At 38°, $k_2=2$ sec.$^{-1}$ and $k_1=7\times10^4$ litre mole^{-1} sec.$^{-1}$. Chance's value of k_1, 10^4 litre mole^{-1} sec.$^{-1}$ at 26°, obtained by a completely different method, is in reasonable agreement. Thorn (37) has applied Slater & Bonner's procedure to the case of malonate, the classical competitive inhibitor of this enzyme, and obtained similar values for the rate constants.

According to the data of Krebs *et al.* (39), the Michaelis constant (K_m) for the oxidation of succinate by frozen and thawed yeast cells is very high (0.01 *M*). A possible explanation is that, although the treated cells have greatly increased permeability to organic acids, the rate of penetration of succinate may still be the factor limiting the rate of its oxidation.

Busch & Potter (41a) have shown that succinate accumulates in the tissues after injection of malonate. Tietze & Klotz (40) and Seaman (41) have studied a number of structural analogues of succinic and malonic acids as inhibitors of succinic dehydrogenase.

Thorn (43) has shown that deuterosuccinate containing 77 atom per cent deuterium in the methylene group was oxidized by the heart muscle succinic oxidase system at about 40 per cent of the rate of normal succinate. The activation energy of the reaction involving the labelled succinate was 1450 ± 450 calories higher and the K_m, 45 per cent higher. These results have implications in the use of deuterium-labelled compounds in studies of intermediary metabolism.

Potter & Reif (38) and Chance (33) have shown that antimycin A in-

hibits respiration by combining with the intermediary factor in scheme E. It is effective in very low concentrations and has been used by both groups to titrate the amount of the factor in heart muscle preparations. Inhibition of the oxidation of α-ketoglutarate, pyruvate, and citrate by antimycin suggests that this factor might be involved in the oxidation of these substrates as well as succinate and DPNH (38).

Bonner (42) has found that a number of metal-binding agents, e.g., histidine, lysine, aspartic acid, denatured globin, 8-hydroxyquinoline, versene, and pyrophosphate activate the succinic oxidase and DPNH oxidase systems in heart muscle preparations. These substances do not act simply by removal of trace metals present in the reagents or enzyme preparations. Inorganic phopshate activates to about the same degree as the metal-binding agents.

Pappenheimer & Williams (44a) have found that diphtheria toxin is most toxic to the *Cecropia* silkworm during those stages of its life cycle when the cytochrome system is functioning. It is much less toxic during diapause when the cytochrome system largely disappears except in the intersegmental muscles; large amounts of toxin affect these muscles but not heart muscle. These findings support Pappenheimer's theory that diphtheria toxin acts by blocking one or more components of the cytochrome system.

Tsou (32) has shown that the succinic oxidase-cytochrome system of adrenal medulla is similar to that in other animal tissues, contrary to earlier reports. Although cytochrome-*c* could not be detected, added ferricytochrome-*c* was reduced in the presence of succinate and added ferrocytochrome-*c* was oxidized. Andrejew & Rosenberg (43a) have shown that the succinic oxidase system in ultrasonic extracts of tubercle bacilli is similar to that in animal tissues. Bueding & Charms (44) have concluded that the respiration of the helminths *Ascaris lumbricoides*, *Litomosoides carinii*, and *Schistosoma mansoni* is not mediated via cytochrome-*c* and cytochrome oxidase, neither of which could be detected in homogenates. They consider that the pigment observed by Keilin (103) in 1925 in *Ascaris* is not cytochrome-*c*, although it closely resembles this compound in its spectroscopic properties.

DPNH oxidase system.—Edelhoch, Hayaishi & Tepley (45) and Mahler, Sarkar, Vernon & Alberty (46) have isolated from pigeon breast muscle and heart muscle, respectively, flavoproteins which react directly with both DPNH and cytochrome-*c*. These enzymes differ from the system studied previously with the Keilin & Hartree heart muscle preparation (31) in that they are not inhibited by BAL.[2] Moreover, the prosthetic group and solubility properties of the heart muscle enzyme are very different from those of Straub's flavo-protein (47) which possesses diaphorase activity[3] but no cytochrome reductase activity. The preparations of Mahler *et al.* have diaphorase

[3] Diaphorase activity is detected by the reduction of dyestuffs such as methylene blue by DPNH. The reactions may be written (*a*) $DPNH+Fl+H^+ \rightarrow DPN^+ + FlH_2$; (*b*) $FlH_2+MB \rightarrow Fl+MBH_2$, where Fl, FlH_2 are flavoprotein and reduced flavo-

activity, which is not surprising since any flavoprotein which is reduced by DPNH should reduce dyes, a relatively nonspecfic reaction for flavoproteins. Green's (3) statement that diaphorase activity is a consequence of the partial inactivation of the reductase enzyme is surprising, since Edelhoch *et al.* (45) found that the ratio of cytochrome reductase to diaphorase activity increased with increasing concentration of alcohol and the higher temperature used during extraction. Moreover, tissue preparations which have received the minimum of treatment possess high diaphorase activity. It is possible that tissues possess two systems for the oxidation of DPNH: first that of Green's group (45, 46), which is a single enzyme reacting directly with cytochrome-*c* and second, the system found in the Keilin & Hartree heart muscle preparations which comprises two components, Straub's flavoprotein and the BAL-sensitive and antimycin-sensitive intermediary factor. This possibility is given support by Potter & Reif's (38) finding of both antimycin-sensitive and antimycin-insensitive pathways for the oxidation of DPNH in heart muscle, liver, and kidney homogenates. Antimycin completely inhibited the oxidation of DPNH by the Keilin & Hartree heart muscle preparation.

Pinchot & Racker (49) found that the DPNH oxidase system of *E. coli* was insensitive to cyanide and to BAL, unlike the heart muscle system (31). The lack of sensitivity to cyanide may indicate that the extract contains an autoxidizable flavoprotein which has been split from the respiratory particles of the organism. The lack of inhibition by BAL cannot, then, be taken as indicating the absence of the BAL-sensitive factor in the DPNH oxidase system of *E. coli*. On the other hand, earlier work of the reviewer (50) showed that extracts of *Azotobacter* contained a very active DPNH oxidase system which was cyanide sensitive but completely insensitive to BAL treatment. The DPNH oxidase system of this organism was also relatively insensitive to azide. Brodie & Gots (50a) have obtained a DPN-cytochrome-*c* reductase in sonic extracts of *E. coli*.

Tissières (51) has obtained from *Aerobacter aerogenes* cell-free preparations of a cyanide-sensitive succinic oxidase system. Very high centrifugal fields (one hr. at 102,000 g) are required to sediment the oxidase system, showing that it must be associated with very small particles.

Reduced triphosphopyridine nucleotide (*TPNH*) *oxidase system.*—Despite the presence of TPNH[2]-specific diaphorases and cytochrome reductases in many tissues and the purification of the latter from liver [Horecker (52)], the enzymic oxidation of TPNH by oxygen has not yet been demonstrated. The Keilin & Hartree heart muscle preparation which oxidizes DPNH very rapidly does not oxidize TPNH at an appreciable rate (50). This is surprising, since isocitrate is oxidized by heart muscle, and heart isocitric dehydrogenase is thought to be a typical TPN-reacting dehydrogenase [Ochoa

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protein and MB, MBH_2 are methylene blue and reduced methylene blue respectively. At infinite methylene blue concentration, the rate of reduction of methylene blue is determined by the rate of reaction (*a*), which is a reaction between naturally occurring substances and may be considered the diaphorase reaction.

(53)]. The report by Plaut & Plaut (54) that the oxidation of citrate by heart muscle preparations is stimulated by nicotinamide mononucleotide, but not by DPN or TPN, is therefore particularly interesting, but further work is required to elucidate the mechanism of this reactivation. Foulkes (54a) has described an unidentified dialysable factor present in boiled yeast juice which catalyses an unknown reaction of citrate in yeast.

TPNH can be oxidized in plant tissues [Mapson & Goddard (55); Conn & Vennesland (56)] and in animal tissues [Rall & Lehninger (57); van Heyningen & Pirie (57a)] and yeast (57) by oxidized glutathione (GSSG). This possibly provides an alternative respiratory chain (Scheme F) to the cytochrome system in plants since enzyme systems are known to be present which catalyze the oxidation of GSH by oxygen.

TPNH→GSSG reductase→GSSG→DHA[2] reductase→DHA→ascorbic oxidase→O_2

Scheme F

Yamaguchi & Joslyn (60a) have purified the DHA reductase of peas 120-fold. This enzyme is specific for GSH. Liver GSSG reductase is not in the mitochondria but in the microsomes or supernatant (57). Thus, if this system were a pathway for the aerobic oxidation of TPNH[2] *in vivo*, the GSH formed in the microsomes would have to be oxidized by the mitochondria, the respiratory granules. Earlier work of Slater (58) shows that added GSH is not oxidized by heart muscle respiratory granules, except under the artificial conditions of adding soluble cytochrome-*c*, because the GSH is not accessible to the cytochrome-*c* bound in the respiratory granules. GSSG reductase is specific for GSSG (57) and TPNH (55, 57). Cystine reductase found by Nickerson & Romano (59) in yeast and peas is, however, specific for cystine and DPNH.

Eddy *et al.* (60) have found that cytochrome-b_1 in *E. coli* is partially oxidized by DHA and have suggested that this cytochrome is a hydrogen carrier involved in the reduction of DHA.

Conn *et al.* (61) have found that two protein fractions from wheat germ extracts are required for the oxidation of TPNH (DPNH was oxidized at one-fourth of the rate). One fraction which contained peroxidase could be replaced by horse-radish peroxidase, and H_2O_2 was identified as an intermediate. The mechanism of the formation of H_2O_2 in this system is not yet known.

Evans & Nason (61a) have found that both flavin-adeninen-diucleotide (FAD) and TPNH are required for the reduction of nitrate by *Neurospora crassa*.

Colowick, Kaplan, and their co-workers (62) have found that extracts of *Pseudomonas fluorescens* and heart muscle catalyze reaction 17.

$$\text{TPNH} + \text{DPN}^+ \rightleftharpoons \text{TPN}^+ + \text{DPNH} \qquad 17.$$

The enzyme (named pyridine nucleotide transhydrogenase) catalyzes a transfer of electrons (and hydrogens) rather than a transfer of phosphate,

since it also catalyzes reaction 18.

$$TPNH + desamino\text{-}DPN^+ \rightarrow TPN^+ + desamino\text{-}DPNH \qquad 18.$$

The enzyme was purified 200-fold (turnover number, 3000 min.$^{-1}$ at 25°C). This enzyme would provide a mechanism for the oxidation of TPNH in the absence of TPN-cytochrome-*c* reductase. The reversal of reaction 17, expected on thermodynamic grounds, was demonstrated only with very low concentrations of TPN^+, since it is a powerful inhibitor. This strong inhibition by one of the products of the reaction recalls the effect of adenylic acid on myokinase (63). One point which remains to be resolved is the abnormal acid lability of both TPN^+ and DPN^+ at the end of reaction 17 when carried out with excess DPN.

α-Glycerophosphate oxidase system.—Lardy *et al.* (63a) have provided new evidence showing that the particulate α-glycerophosphate dehydrogenase of rabbit muscle is a different enzyme from the soluble protein involved in the glycolytic cycle. Unlike the latter, it is not a pyridine nucleotide enzyme. By freeing the enzymes from triosephosphate isomerase, it was shown that dihydroxyactonephosphate is the oxidation product of both enzymes.

Cytochrome oxidase and cytochrome–a.—Ball *et al.* (66) have concluded from a study of the effect of carbon monoxide on the spectra of desoxycholate-dispersed heart muscle preparation that cytochrome oxidase and cytochrome-a_3 are identical, in agreement with the view expressed by Keilin & Hartree (19) in 1939. The observations of Ball *et al.* were very similar to those of Straub (67). The measurements of Keilin & Hartree, Straub, Chance and Ball *et al.* are all essentially in agreement except for the position of the γ-band of the carbon monoxide compound, which Ball *et al.* place at 423 mμ, compared with 430 to 432 mμ by the other workers; the latter value is in agreement with the photochemical absorption spectrum obtained in Warburg's laboratory (68). Chance ascribes the discrepancy to the presence of haemoglobin in Ball's preparations. Ball *et al.* suggest that the a band of cytochrome at 605 mμ is due to a complex compound of four iron porphyrin rings in close juxtapostion, only one of which can react with oxygen or carbon monoxide. After this reaction, all four of the iron atoms might then become oxidized to the ferric form, with the simultaneous reduction of one molecule of oxygen to two molecules of water. An interesting finding of Ball *et al.* is that the addition of soluble cytochrome-*c* increased the rate of reduction of cytochromes $(a+a_3)$ by ascorbate or succinate, but not by $Na_2S_2O_4$. (The enzyme preparation required cytochrome-*c* for the oxidation of succinate.) They consider that this shows that cytochrome-*c* reduces cytochrome-a_3, but the rate of the reduction of cytochrome-a_3 is not stated. This is important since complete reduction should require only a fraction of a second if this reaction is, in fact, involved in the oxidation of soluble cytochrome-*c*. The direct reduction of cytochrome-*a* or a_3 by reduced cytochrome-*c* in the absence of any other hydrogen donor has not yet been demonstrated.

The detailed mechanism of reaction 19 is still highly speculative ($a_3^{\cdot\cdot}$, $a_3^{\cdot\cdot\cdot}$ = ferro- and ferricytochrome-a_3 respectively).

$$4\,a_3^{\cdot\cdot} + O_2 + 4\,H^+ \rightarrow 4\,a_3^{\cdot\cdot\cdot} + 2\,H_2O \qquad 19.$$

Studies such as that of George & Stratmann (69) on the kinetics of the oxidation of myoglobin to metmyoglobin may eventually provide a clue. It may be necessary to introduce the phosphorylation of ADP into this reaction (see p. 35).

Wainio *et al.* (76) found at least four components when heart muscle preparation dispersed in desoxycholate was examined in the analytical ultracentrifuge, the slowest sedimenting being probably cytochrome-*c*. What they call "cytochrome-*b*" (on the basis of spectrum only, which is close to that of denatured protein haemochromogen) sedimented slightly more rapidly than cytochrome oxidase.

Lemberg & Falk (70) have compared the properties of haem-*a*,[4] prepared by the method of Rawlinson & Hale (71), and the related porphyrin with those of a number of haems and porphyrins of known structure containing carbonyl groups in the side chain. The comparison supported previous evidence for the presence of a formyl side chain. Structures for porphyrin-*a* which are consistent with all the evidence available are 4-ethyl-2-formyl-1,3,5,8-tetramethylporphin-6-acrylic acid-7-propionic acid and a closely related compound. Falk & Rimington's (72) attempts to isolate porphyrin *a* in amounts suitable for chemical studies failed. All preparations were mixtures of closely related substances, which are formed during the isolation procedure by the action of acid on a precursor. Kiese (72a) also comments on the instability of porphyrin-*a* but claims to have found conditions for the isolation of the unchanged porphyrin, from which haem-*a* can be reformed.

Chance's (33) reference to "current ideas on the uniformity of the properties of the 'Oxygen-transferring Enzyme' " is difficult to understand, since it has been known for over 25 years that many microorganisms have a cytochrome system different from that found in bakers yeast or animal tissues. In particular, the typical ($a+a_3$) band in the red is replaced by the bands of cytochromes-a_1 and a_2. Tissières (73) has studied the system in *Aerobacter aerogenes* which has a particularly strong a_2 band. Since cyanide and carbon monoxide produced changes in the a_2 band comparable to those previously found with cytochrome-a_3 (19), while cytochrome-a_1 was not affected, Tissières concluded that cytochrome-a_2 is the terminal oxidase in this organism. Chance's (33) study of the γ-bands of this organism confirms the absence of cytochrome-a_3 [see also Smith (73a)]. Later, however, Tissières (51) obtained small-particle preparations which oxidized succinate and contained cytochromes-b_1 and a_1 but no detectable cytochrome-a_2. It is possible, however, that a concentration of a_2 sufficient for measurable oxidation might not be visible spectroscopically, as Tissières suggests.

[4] Haem-*a*, the haem of cytochrome-($a+a_3$), has been named cytohaemin by Warburg & Gewitz (70a).

Chin (74) has concluded that cytochrome-a_2 is also the terminal oxidase in *Acetobacter peroxidans*. He has also identified a new cytochrome-*a*, named a_4, which has the α-band at 612 mμ.

Hochster & Quastel (77) have studied the use of MnO_2 as an artificial hydrogen acceptor. Since it oxidizes ferrocytochrome-*c* directly, but not DPNH or flavoprotein, it can substitute for cytochrome oxidase. A suspension of MnO_2 is used, so that its concentration in solution remains constant. Brodie & Gots (78) have found that nitrofurans, e.g., furacin, act as hydrogen acceptors, reacting with diaphorase. Kun (79) has shown that reduction of the dye triphenyl tetrazolium chloride by tissues takes place by reaction with flavoproteins.

Cytochrome-c.—Theorell *et al.* (80), continuing their studies on the chemistry of cytochrome-*c*, have found that in strongly acid solution cytochrome-*c* combines with two protons ($pK_1 = 2.49$, $pK_2 = 1.99$) to give the compound previously named as spectroscopic type I. A new type of cytochrome-*c*, containing two chlorine atoms, was identified in acid solution.

The purification of cytochrome-*c* by ion-exchange resins has been studied by Neilands (81), Paléus (82), and Margoliash (83). Margoliash describes simple procedures using ion-exchange resins, for preparing concentrated solutions of cytochrome-*c*, containing 0.43 per cent iron, in either the ferri- or the ferro- form.

Davenport (84) has obtained mesoporphyrin by treatment of cytochrome-*c* with sodium amalgam followed by extraction with acetic acid-ether mixture. Under these conditions, protohaematin yielded only protoporphyrin, while haematoporphyrin gave mesoporphyrin. These observations support Theorell's view (85) that side-chains 2 and 4 of the haem of cytochrome-*c* are involved in the stable haem-protein linkage.

A kinetic investigation of the slow reaction between ferricytochrome-*c* and cyanide between pH 6 and 9 by George & Tsou (86) suggested that both HCN and CN^- react. Calculations of the entropy changes from measurements of the heats of activation support a model for ferricytochrome-*c* in which the haematin iron atom is firmly bound on both sides of the protein (cf. 85).

Tsou (87) has shown that cytochrome-*c* firmly bound to particles of the Keilin & Hartree heart muscle preparation does not combine with cyanide. Although it has been recognized for some time that this bound (endogenous) cytochrome-*c* is more active than cytochrome-*c* in solution, this is the first qualitative difference found. A significant finding of Tsou is that salt-washed heart muscle mince, which is deficient in cytochrome-*c*, will take up cytochrome-*c* from salt-free solutions and that this cytochrome-*c* reincorporated into the heart muscle mince behaves in all respects like the original bound cytochrome-*c*, including the lack of reaction with cyanide. This shows that isolated cytochrome-*c* is not an irreversibly modified form of the natural pigment as has sometimes been supposed.

Localization of respiratory chain in cell.—Cleland & Slater (88) have

shown that the cytoplasmic granules, studied by cytologists for nearly a hundred years under the names interstitial granules, mitochondria, or sarcosomes, contain most and probably all the respiratory activity of heart muscle. In this respect they correspond to the mitochondria of liver and kidney cells. Also like mitochondria, sarcosomes contain a semipermeable membrane which acts as an osmotic barrier. Cleland (88) has measured the permeability to a number of substances by determining the rate of swelling of the sarcosomes by a light-scattering method [see also Raaflaub (88a)]. Sucrose, KCl, and the substrates succinate, fumarate, and malate penetrate very slowly, whereas ethylene glycol, glycerol, and $HPO_4^{=}$ penetrate rapidly. From morphological studies it is concluded that the respiratory chain is situated in the sarcosome membrane. When shaken, swollen sarcosomes obtained in highly hypotonic media fragment into pieces of membrane and soluble protein. It is believed that the Keilin & Hartree preparation consists of fragmented sarcosomal membrane. Hogeboom & Schneider (89) found that treatment of liver mitochondria with sonic oscillations causes disruption into soluble protein and polydisperse particulate material containing cytochrome-*c*, cytochrome oxidase, and DPN[2]-cytochrome-*c* reductase. This process probably has essentially the same result as shaking swollen sarcosomes, except that the membrane would be broken into smaller particles.

Harman & Feigelson (90) have also identified the respiratory granules of heart muscle with the mitochondria. Paul & Sperling (91) have shown a correlation between "cyclophorase" (respiratory) activity and mitochondria density of different types of striated muscles. Lawrie (92) has found a correlation between the myoglobin content and the activity of the cytochrome system in different types of muscles. Chappell & Perry (93) found that the respiratory activity of rabbit skeletal and pigeon breast muscles was mainly confined to the heavy granules.

Brody & Bain (93a) have shown that brain mitochondria have activities similar to those in other tissues, except that fatty acids are not oxidized.

Respiratory chain in plants.—Bonner *et al.* (65) have shown that mitochondria isolated from mung bean oxidize pyruvate to CO_2 and water, apparently via the Krebs cycle. Hydrogen transfer proceeds through the cytochrome system, and oxidative phosphorylation was shown by the incorporation of P^{32} into ATP [see also Vishniac (94)]. Thus these plant mitochondria appear to be completely comparable with those in animal tissues.

Davenport & Hill (94a) have isolated cytochrome-*f*, which is found in chloroplasts, from parsley leaves. The spectrum of ferrocytochrome-*f* resembles that of ferrocytochrome-*c*, but the bands are sharper and are shifted towards the red. The oxidation-reduction potential (E'_0) is higher than that of other cytochromes (0.365 volt at pH 6.6 to 7.7 and 30° C). Davenport & Hill suggest that cytochrome-*f* may be concerned in the photosynthetic oxidation-reduction reactions in the chloroplast. Hill *et al.* (94b) have found that leaf extracts contain a soluble factor, probably a protein, which is reduced directly by illuminated chloroplasts with the evolution

of oxygen. The reduced factor was detected by its ability to reduce methaemoglobin.

Ducet & Rosenberg (95) have shown that a partial pressure of carbon monoxide insufficient to inhibit the respiration of intact leaves promotes an aerobic glycolysis. This is very similar to the effect found by Laser (95a) with animal tissues.

By means of special spectrophotometric techniques, Lundegårdh (64) has studied the cytochrome system in intact wheat roots. The system appears to be similar to that in animal tissues, although there are important differences, for example the γ band of cytochrome-$a+a_3$ (mainly due to a_3 (19)) does not disappear in the presence of cyanide and air. The rapid inhibition by cyanide and the ability of living roots slowly to oxidize reduced cytochrome-*c* suggests to Lundegårdh that the cytochrome system of wheat roots is localized in or near the surface of living protoplasm, perhaps as a macromolecular component of the protoplasmic membrane. Bonner *et al.* (65), on the other hand, believe that the cytochrome system in mung bean is localized in mitochondria as in animal tissues. Lundegårdh calculates the oxidation-reduction potentials of the cytochromes from the variation of the oxidation-reduction balance of the individual cytochromes. His value for cytochrome-*b* (+0.14 to 0.17 volt) is much higher than that found by Ball (35) in animal tissues (−0.04 volt). But it must be remembered that in the intact cell other reactions involving energy-rich phosphate will alter the position of equilibria between hydrogen or electron carriers and therefore appear to alter the oxido-reduction potential (see p. 39). The oxidation of reduced cytochrome-*c* by fumarate also observed by Lundegårdh could hardly operate without a source of energy.

Oxidative Phosphorylation

Mechanisms.—The most important development during the year concerning the mechanism of oxidative phosphorylation was the clarification of the "substrate-linked" phosphorylation associated with the oxidation of α-ketoglutarate (see p. 22). It is very probable that an intermediate phosphorylated compound is formed in reaction 14 before the formation of ATP,[2] but this has not yet been identified. Succinylphosphate and CoA-pyrophosphate (the probable intermediate in the activation of acetate; see p. 45 to 46) have been eliminated as intermediates by Kaufman (96), who considers that the intermediate is probably CoA-monophosphate, but there is, as yet, no direct evidence.

This substrate-linked phosphorylation accounts for only one of the 15 molecules of ATP synthesized from ADP during the oxidation of pyruvate to CO_2 and water (cf. 4a, 97). Ochoa & Stern (4a) believe that the remaining 14 are derived as follows: (*a*) 3×3, from the aerobic oxidation of DPNH (if DPN is a component of the pyruvic and α-ketoglutaric oxidase systems, this step is repeated three times in the citric acid cycle); (*b*) 3 from the aerobic oxidation of TPNH; (*c*) 2 from the aerobic oxidation of succinate to fuma-

rate. Phosphorylation coupled with the oxidation of TPNH has not yet been directly demonstrated and it is difficult to isolate the succinate→fumarate step in enzyme preparations which carry out oxidative phosphorylation. We are still completely ignorant of the mechanism of these phosphorylations, which may be called respiratory chain phosphorylations to distinguish them from the substrate-linked phosphorylation.

Lindberg & Ernster (98) found that small amounts of inorganic pyrophosphate accumulated during the oxidation of α-ketoglutarate by heart-muscle mitochondria in the presence of inorganic phosphate, hexokinase, and either adenosinemonophosphate (AMP) or ADP. They propose that pyrophosphate is formed in a side reaction by the breakdown of a primary ester, which is responsible for most of the ATP formation by phosphorylating ADP in a reaction called the "primitive mechanism." Another pathway, the "differentiated mechanism," involves the renewal of primary phosphate carrier by phosphorylation of AMP (but, see p. 36). This explanation is at variance with previous work (99), which indicated that inorganic pyrophosphate was formed by the breakdown of ATP. The conclusions of Lindberg & Ernster are entirely dependent upon the presence of sufficient hexokinase in their experiments to react with all the ATP formed. No evidence is presented that this was the case.

Kaplan (99a) has studied the chemistry of the reaction between DPN^+ and phosphate from the point of view of the possible mechanism of the phosphorylation associated with the enzymic aerobic oxidation of DPNH. There is no spectroscopic evidence of compound formation between DPN^+ and phosphate, but phosphate increases the rate of destruction of DPN^+ by heat. Kaplan speculates on the possibility that phosphorylation accompanies the oxidation of DPN^+ to a pyridone, from which DPN is regenerated by reaction with DPNH.

Methods.—The usual method of measuring the yield of oxidative phosphorylation is to determine the disappearance of inorganic phosphate. This has obvious disadvantages for studying the kinetics of the early stages of the reaction and for such problems as measuring the phosphorylation associated with the stoichiometric reaction of a respiratory catalyst (100). Slater (101) has described procedures which depend upon sensitive enzymic methods of measuring the phosphorylated products, e.g., hexosemonophosphate (HMP) and ATP. Possible errors as a result of known side reactions have been examined, and it is concluded that, if sufficient hexokinase is used to compete with the adenosinetriphosphatase, errors due to known dephosphorylation reactions are negligible. The P:O ratios found truly represent the phosphorylative activity of the preparations and should not be raised to allow for losses.

Slater & Holton (101) have found that phosphorylation proceeds at a uniform rate from the moment of adding the enzyme preparation without any appreciable lag. The lag previously found (100) in the synthesis of HMP is due to the formation of the steady-state concentration of ATP dictated by the reactions:

$$ADP + H_3PO_4 \rightarrow ATP + H_2O \text{ (oxidative phosphorylation)} \qquad 20.$$

$$ATP + \text{glucose} \rightarrow HMP + H_2O \text{ (hexokinase)} \qquad 21.$$

$$\text{glucose} + H_3PO_4 \rightarrow HMP + H_2O \qquad \text{(overall reaction)}$$

There is no lag when (ATP+HMP) is measured or if hexokinase is omitted and ATP formation measured. In the presence of a very large amount of hexokinase, HMP is formed with a negligible time lag.

Location of phosphorylation in the respiratory chain.—This subject has been ably reviewed by Hunter (97). Copenhaver & Lardy (105) obtained a P/O ratio approaching 2 with α-ketoglutarate as substrate and methylene blue as hydrogen acceptor. This is the first time oxidative phosphorylation has been obtained with methylene blue, a reagent which is particularly useful for locating portions of the respiratory chain. In agreement with previous work, phosphorylation was also obtained with ferricyanide as hydrogen-acceptor. With β-hydroxybutyrate as substrate, Copenhaver & Lardy obtained the same P:2 ferricyanide ratio in the absence as in the presence of antimycin which completely inhibited oxygen uptake. They conclude that "the electron carrier or carriers donating electrons to ferricyanide are proximal to that factor inhibited by antimycin A." In agreement with Judah (102) [see also Lehninger (48)], Copenhaver & Lardy found small uptakes of phosphate during the oxidation of ascorbic acid by cytochrome-*c* and mitochondria with P/O ratios approaching 0.5. This may indicate that the cytochrome oxidase reaction is coupled with phosphorylation, but this has not yet been directly demonstrated with reduced cytochrome-*c* as substrate in the absence of ascorbic acid. Direct experiments of this type as well as further experiments with cytochrome-*c* as hydrogen acceptor (100) are required to settle the question whether or not all the phosphorylation occurs between substrate and cytochrome-*c*. Thermodynamic arguments (48, 4a) can never be decisive without a knowledge of the steady-state concentrations of the reactants.

Burton (104) and Oesper (104a) have both lowered the value for the standard free energy change in the hydrolysis of ATP. Burton's new value, derived from free energy data for glycerol and pyruvate and equilibrium data for the enzymic conversion of glycerol to triosephosphate and subsequently to pyruvate, is $-9{,}200 \pm 800$ cal. at pH 7.4 (cf. 12,000, the usually accepted value). Oesper's new value, obtained by a recalculation of Meyerhof's data on the triosephosphate dehydrogenase reaction, is $-10{,}500$ cal., at pH 7.4 and 20°C. Burton has calculated that at *in vivo* concentrations of ADP, ATP, and inorganic phosphate, the free energy change is probably 13,000 to 15,000 cal.

Copenhaver & Lardy (105) and Krebs (106) have provided the experiments on which the recent assessments of the yields (4a, 97) in the different steps of the tricarboxylic acid cycle have been largely based. The results obtained by Copenhaver & Lardy with liver mitochondria are summarized in Table I, the last column of which gives the values assigned by the authors to the various steps. The ratio of 4 for the oxidation of α-ketoglutarate to

TABLE I

P/O Ratios Obtained by Copenhaver & Lardy (105) in Liver Mitochondria

Reaction	Mean Measured P/O	Probable P/O
(1) α-Ketoglutarate→succinate	3.4	4
(2) 2 pyruvate→acetoacetate	2.6	3
(3) β-hydroxybutyrate→acetoacetate	2.5	3
(4) Succinate→	1.7	2
(5) Citrate→α-ketoglutarate	2.6	3
α-ketoglutarate→succinate		4
(6) Glutamate→α-ketoglutarate	2.6	3
α-ketoglutarate→succinate		4

succinate is in agreement with previous values obtained by Judah (102) and Krebs (106). Slater & Holton (30), on the other hand, find that the P/O ratio in heart muscle sarcosomes (mitochondria) for this step is only 3. The reason for the discrepancy is not known. It is unlikely to be a true tissue difference since Feldott & Plaut (106a) have obtained approximately the same values in heart muscle as Copenhaver & Lardy found in liver. Since the unknown anaerobic reaction of α-ketoglutarate (see p. 23), stimulated by crude hexokinase preparations, is accompanied by phosphorylation, these preparations can cause the P/O ratio to be overestimated (30). The correspondence between oxygen uptakes and α-ketoglutarate disappearance show that this error was not operating in Copenhaver & Lardy's experiments.

Adeninenucleotide specificity.—As indicated in last-year's review (4a), it is now agreed that ADP is phosphorylated by respiring tissue preparations more rapidly than AMP. The earlier work did not, however, exclude the possibility that AMP, as well as ADP, might be directly phosphorylated by the oxidative phosphorylation reaction itself. Lindberg & Ernster (98) believe that this is, in fact, the case and that this phosphorylation of AMP is indispensable for the overall reaction. The direct testing of this possibility required a preparation with very little myokinase activity, such as cat heart sarcosomes. (Liver mitochondria are rich in myokinase.) Slater & Holton (101) have found with a very dilute preparation of cat sarcosomes that AMP was not phosphorylated at a significant rate under conditions where ADP was rapidly phosphorylated. The rapid phosphorylation of AMP by liver mitochondria must depend upon the formation of ATP from endogenous ADP, followed by the myokinase reaction [cf. Kielley & Kielley (107)]. Eggleston & Hems (108) have determined the equilibrium constant of the myokinase reaction by measuring the concentrations of ATP, ADP, and AMP at equilibrium. The mean value of $K = [ATP]\,[AMP]/[ADP]^2$ was 0.44 at pH 7.4 and 25°, a value close to that found by Kalckar (109).

Control of respiration by phosphorylation.—Last year has seen further studies (cf. 4a) of the stimulating effect of acceptors of energy-rich phosphate on the rate of respiration. Rabinovitz *et al.* (110) found that the rate of oxidation of pyruvate and oxalacetate was increased by hexokinase and glucose. Using rat-liver mitochondria prepared in sucrose and containing very little adenosinetriphosphatase, Lardy & Wellman (111) found that the rate of oxidation of a number of substrates in the presence of magnesium, ATP and inorganic phosphate is very low unless phosphate acceptor systems, such as hexokinase-glucose or creatinephosphokinase-creatine are added. Dinitrophenol stimulated to the same extent [see also Potter & Recknagel (113)]. Lardy & Wellman offer the following physiological interpretation.

The lowest rate of oxygen consumption by an organ *in situ* probably reflects the energy requirements of that organ for its "basal" metabolic functions and the maintenance of its structural integrity. When the organ is stimulated to do additional work (secretion, contraction, synthesis), inorganic phosphate is liberated from ATP, creatine phosphate, or other energy sources. The increased quantity of inorganic phosphate will . . . cause glycogen to be converted to hexosemonophosphate. This provides a substrate for the glycolytic machinery. The increased quantities of intracellular phosphate and acceptors also permit the oxidative enzymes to attack the pyruvate made available by glycolysis. The oxidative reactions in turn fix the inorganic phosphate in the form of high-energy phosphate compounds . . . . When the stimulus is withdrawn, respiration continues at the elevated rate only until the organ is restored to its original state . . . . With no compounds remaining in the unphosphorylated state, the respiration machinery stops with the various enzymes blocked in the acylated and/or phosphorylated states . . . . Glycolysis will have been effectively stopped because of the low hexose phosphate concentration and the low inorganic phosphate and acceptor concentrations.

McIlwain (112) has expressed similar views. The experiments of his group provide an excellent example of the effect of a stimulus on the metabolism. When brain slices are electrically stimulated, creatinephosphate levels fall and inorganic phosphate rises, while at the same time respiration and glycolysis are increased. Substances which lowered the creatinephosphate level (e.g., dinitrophenol) caused some increase in respiration but abolished the effect of stimulation, so that the stimulated slice in the presence of dinitrophenol had lower rates of respiration and aerobic glycolysis. Kratzing (112a) has found similar increases of respiration and aerobic glycolysis to occur during electrical stimulation of rat diaphragm but none with kidney slices and only a slight effect with lung.

The biochemical mechanism of the effect of phosphate acceptors on the rate of respiration has not been completely clarified by Lardy or by work done previously. There are two possibilities. (*a*) The adeninenucleotides are completely phosphorylated so that the steady-state concentration of ADP is very low; the oxidation might then fail for purely stoichiometric reasons since ADP is an essential requirement for the oxidation. (*b*) Some of the phosphorylation reactions are readily reversible and the back reaction be-

comes important when [ATP]/[ADP] is high. The high yield of phosphorylation associated with the reduction of cytochrome-*c* by α-ketoglutarate (100) indicates that the overall reaction and hence the individual phosphorylation steps should be reversible. The reversibility of the "substrate-linked" phosphorylation was shown by Kaufman (23) (see p. 23).

Effect of hormones, drugs, and vitamin E.—The characteristic symptoms of hyperthyroidism have promoted much speculation that the action of the thyroid hormone is to uncouple respiration from phosphorylation with the net result that foodstuff will be rapidly consumed without utilization of the energy which will appear as heat. Lardy (114) proposes, as a general theory, that hormones may act to uncouple a phosphorylation step immediately preceding a rate-limiting reaction. Thyroxine or better still 3,5,3′-triiodothyronine, the more active compound isolated by Gross & Pitt-Rivers (115) from thyroid are examples. Martius (116) also finds that thyroxine acts as an uncoupling agent. Lipmann and his co-workers (117), on the other hand, were unable to find any striking effect on the P/O ratio with thyroxin *in vitro* or with preparations from thyroxin-treated rats. Moreover, the antithyroxin drug (3,5-diiodo-4-benzyloxybenzoic acid) was an uncoupling agent. Aebi (118) has shown that the effect of hyperthyroidism is to increase the number of mitochondria, not their activity. The increase of the mitochondrial fraction (30 per cent) exactly paralleled the increase of the specific activity of succinic dehydrogenase in the homogenate.

Weil-Malherbe's (119) suggestion that the increased respiration of dystrophic muscle in vitamin E deficiency might be due to a selective inhibition of the energy-coupling mechanism is very similar to Lardy's theory for the action of thyroxine. However, Rabinovitz & Boyer (120) have found no difference in the phosphorylative abilities of hearts from dystrophic and control rabbits.

Brody & Bain (121) have found that barbiturates uncouple phosphorylation from respiration. Appreciable effects were obtained at concentrations of drugs which approximate those which are necessary *in vivo* to produce surgical anaesthesia. However, *in vivo*, pentobarbital sodium [nembutal; sodium 5-ethyl-5-(1-methylbutyl)barbiturate] increases the phosphocreatine of the brain at the expense of inorganic phosphate (112).

Stability of oxidative phosphorylation.—A long recognized difficulty in the study of the enzyme systems which bring about oxidative phosphorylation is their great instability at temperatures more than a few degrees above freezing, especially in the absence of the reaction mixture used to demonstrate oxidative phosphorylation. Slater & Cleland (88) have now shown that this instability, at least in heart sarcosomes where it is particularly marked, is due to calcium in the preparations. It can be prevented by the addition of the calcium-binding agent, ethylenediaminetetraacetic acid (versene), which has no inhibitory effect on oxidative phosphorylation. The component of the reaction mixture which, in the absence of versene, confers limited stability is ADP (cf. 107). This can be replaced by ATP, but not by AMP. The simul-

taneous addition of magnesium, fluoride and phosphate also gives some protection. The mechanism of the effect of calcium is obscure; it may cause a loss of bound energy-rich phosphate compounds. Morphological changes in the sarcosomes which also occur on incubation can be prevented by versene, ATP, or ADP, suggesting that a source of energy is necessary to maintain the structure of these granules, as has also been proposed by Harman & Feigelson (90).

Reversal of oxidative phosphorylation.—It is often stated that photosynthesis is, in its overall effect, the reverse of respiration. This is correct insofar as carbon and hydrogen are concerned. But a more complete account of respiration is given by reaction 22:

$$\text{foodstuff} + O_2 + n\ \text{ADP} + n\ H_3PO_4 \rightarrow CO_2 + H_2O + n\ \text{ATP} \qquad 22.$$

Since so far as we know at present at least the major portion of the energy of respiration which can be utilized by the cell appears as ATP, it is obvious that a reversal of reaction 22 would not achieve any gain to the cell, unless the light reaction itself is able to generate$\sim$P. In the absence of any evidence for this, it would appear likely that photosynthesis is not simply a reversal of the respiratory chain but that it proceeds by a different mechanism. This does not exclude the possibility that some of the individual reactions in photosynthesis might be the reverse of those in respiration, e.g.,

$$\text{ATP} + X + YH_2 \rightarrow \text{ADP} + H_3PO_4 + XH_2 + Y \qquad 23,$$

where X and Y are adjacent hydrogen-carriers in the respiratory chain (e.g., X = pyridinenucleotide, Y = flavoprotein). The ATP could be provided by oxidative phosphorylation in other granules, the mitochondria. This would provide a possible chemical mechanism for Hill's postulate in 1939 (123) that the initial reduction by the chloroplasts does not split water to the extent necessary to reduce carbon dioxide to the level of carbohydrate, so that oxygen must be consumed again to give the necessary energy for the process [see also Warburg (124)].

During the last year, Vishniac & Ochoa (94) showed simultaneously with Tolmach (125) and Arnon (126), that chloroplast grana preparations bring about the reduction of TPN and DPN in the light. The reduction has not yet been directly observed but the formation of reduced nucleotide was detected by the addition of enzyme systems known to react with these compounds, e.g.,

$$\text{TPN}^+ + H_2O \xrightarrow{\text{light}} \text{TPNH} + H^+ + \tfrac{1}{2}O_2 \qquad 24.$$

$$\text{TPNH} + CO_2 + \text{pyruvate} + H^+ \rightarrow \text{TPN}^+ + \text{malate} \qquad 25.$$

$$CO_2 + \text{pyruvate} + H_2O \xrightarrow{\text{light}} \text{malate} + \tfrac{1}{2}O_2 \qquad \text{(overall reaction)}$$

This may be one of the reactions leading to the fixation of CO_2 in photosynthesis. Ochoa & Vishniac also obtained small amounts of succinate when chloroplasts were irradiated in the presence of fumarate and an *E. coli* ex-

tract. This need not be fundamentally different from the other reactions described, since reaction 26

$$DPNH + fumarate + H^+ \rightarrow DPN^+ + succinate \qquad 26.$$

is known in animal tissues (31). Catalytic amounts of DPN could have been supplied by the *E. coli* extract or the chloroplasts.

Davies & Krebs (127) have also postulated a reaction of the general type of reaction 23 to account for the secretion of H^+ ions. In their scheme, X is flavoprotein and YH_2 is ferrocytochrome-*c*.

Pyridine Nucleotide Dehydrogenases

Fisher *et al.* (127a) have shown that deutero-DPN, prepared by the reduction of DPN[2] by α-dideuteroethanol in the presence of alcohol dehydrogenase, lost all its deuterium on reoxidation with acetaldehyde or with pyruvate (in the presence of lactic dehydrogenase). In the latter case, one atom of deuterium was found in the lactate. Chemically-reduced DPN lost only half its deuterium. This shows that in the enzymic reaction the deuterium is transferred to only one side of the plane of the pyridine ring. These authors conclude that the enzymic oxido-reduction catalyzed by alcohol of lactic dehydrogenases cannot involve formation of an intermediate which exchanges hydrogen with the medium. Transfer of hydrogen through a sulphydryl group in these enzymes is thought to be improbable.

Glyceraldehydephosphate dehydrogenase.—This important enzyme is being actively investigated in a number of laboratories. Harting & Velick (128) have shown that it catalyzes the oxidation of acetaldehyde in the presence of inorganic phosphate to acetylphosphate. Unlike extracts of *C. Kluyveri* (2), CoA is not required for this reaction, but acetyl-CoA is formed on the addition of CoA. Similarly acetyl-glutathione can be synthesized by the enzyme. They conclude, therefore, that an acyl enzyme complex (cf. Racker) occurs as an intermediate. It is generally accepted that CoA is not involved in the action of this enzyme *in vivo*. The fact that it can form acetyl-CoA when CoA is added indicates that some caution is desirable when interpreting the effects of added CoA[2] (or GSH) in other systems.

Racker (129) has found glutathione (GSH) in the extract obtained by acid and heat denaturation of the purified glyceraldehydephosphate dehydrogenase; GSH was identified by three independent enzymic methods and by paper chromatography of the reduced and oxidized forms. Racker believes that GSH is a firmly bound prosthetic group of the enzyme. Since, however, GSH is a tripeptide, one might consider that it is part of the protein molecule. The distinction is probably not important, since it is the -SH group of the cysteine moiety which is thought to be involved in the catalytic reaction. Racker found that when DPN was added to the pure reduced enzyme, freed from bound DPN, an absorption band with a broad maximum at 365 mμ was obtained; this is ascribed to an enzyme-DPN

complex. Racker believes that the -SH group on the enzyme combines with the DPN, since combination of the -SH group with p-chloromercuribenzoate prevents the complex formation. This is also consistent with Rapkine's (130) earlier finding that DPN protects this enzyme against inactivation by -SH combining agents. Racker suggests that the first step of the action of the enzyme is a cleavage of this sulphur-DPN bond by the aldehyde group of the substrate to give DPNH and a thiol ester of the enzyme. This mechanism implies that DPNH is not firmly bound to the enzyme, which might explain why a protein-DPNH complex of the type found by Theorell & Bonnichsen (131) with liver alcohol dehydrogenase has not yet been identified with glyceraldehydephosphate dehydrogenase. Racker earlier (22) proposed that diphosphoglyceric acid was formed by phosphorolysis of this thiol enzyme where $E \cdot SH$ is the free enzyme and $R \cdot COO{\sim}H_2PO_3$ is diphospho glyceric acid:

$$E \cdot S \sim CO \cdot R + H_3PO_4 \rightleftharpoons E \cdot SH + R \cdot COO \sim H_2PO_3 \qquad 27.$$

There are still some puzzling observations of both Racker and Harting & Velick which are not explained by Racker's mechanism. Racker finds that DPN is required for the arsenolysis of diphosphoglyceric acid, while Harting & Velick have found that it is necessary for the exchange between acetylphosphate and P^{32} which is also catalyzed by this enzyme; the exchange rate is many times faster than the oxidation and reduction of DPN.

Racker does not speculate on the nature of the DPN-thiol linkage, which gives the absorption maximum at 365 mμ. It is noteworthy that Colowick *et al.* (132) obtained no evidence of the formation of a complex between DPN and GSH. DPN "bound" to glyceraldehydephosphate dehydrogenase is still able to react with a number of enzymes. Astrachan (132b) has found that it reacts at one-half the rate of free DPN with various hydrolytic enzymes, one-quarter the rate with deaminase, and one-fifth the rate with alcohol dehydrogenase.

Bücher & Garbade (132a) conclude from a study of the effect of arsenate concentration on the enzymic activity that it is unlikely that the enzyme reaction is preceded by the spontaneous combination of phosphoglyceraldehyde with arsenate. Nygaard & Sumner (134) have found that this enzyme is inhibited by bis(diethylthiocarbamyl)disulfide (antabuse; tetraethylthiuram disulfide) like other enzymes which oxidize acetaldehyde.

Glutamic dehydrogenase.—Olson & Anfinsen (133) have described the crystallization and physical characterization of liver glutamic dehydrogenase. It has a molecular weight (10^6) unusually high for a protein with a simple single enzymic activity. At the pH optimum the relative rates of reduction of pyridinenucleotides by glutamate were: DPN(100), desamino-DPN (60), and TPN (35). The enzyme is completely specific for glutamate and ammonia. Large variations of ammonia concentration had little effect on the equilibrium constant, which indicates that if α-iminoglutarate is an inter-

mediate, it is not present in appreciable concentrations. No spectroscopic evidence of compound formation between the enzyme and DPN or DPNH was obtained.

L-amino acid dehydrogenase.—Nisman & Mager (135) have shown that the reduction of DPN by a number of L-amino acids in the presence of cell-free extracts of *Clostridium sporogenes* requires inorganic phosphate or arsenate. No uptake of inorganic phosphate could be demonstrated.

Glucose dehydrogenase.—Ox liver glucose dehydrogenase has been purified 125-fold by Strecker & Korkes (136). The enzyme is active with both DPN and TPN. As with the flavoprotein, notatin (137), the primary oxidation product is gluconolactone, thus:

$$\text{glucose} + \text{DPN}^+ \rightleftharpoons \text{gluconolactone} + \text{DPNH} + \text{H}^+ \qquad 28.$$

$$\text{gluconolactone} + \text{H}_2\text{O} \rightleftharpoons \text{gluconic acid} \qquad 29.$$

$$\text{gluconic acid} \rightleftharpoons \text{gluconate}^- + \text{H}^+ \qquad 30.$$

Because reaction 30 is nearly irreversible at neutral pH, the overall reaction is practically irreversible. However, by using gluconolactone as reactant, Strecker & Korkes were able to show that reaction 28 is reversible with an equilibrium constant of 15 at pH 6.7. Near the pK of gluconic acid (3.78) it was possible to show that the overall reaction was reversible. From the various equilibria it was calculated that E'_0 of glucose$\rightleftharpoons$gluconate is -0.43 volts at pH 7.0, in exact agreement with earlier determinations. It would be interesting to know if both α and β-glucose react with glucose dehydrogenase or if it is specific for one form, like notatin (see p. 44).

Glucose-6-phosphate dehydrogenase.—O. Cori & Lipmann (138) have shown that the primary oxidation product formed in the reaction catalyzed by yeast glucose-6-phosphate dehydrogenase is a compound which reacts with hydroxylamine to form hydroxamic acid. The rate of reaction with hydroxylamine is similar to that of δ-gluconolactone and much slower than acetylphosphate. Cori & Lipmann concluded that the pyranose form of glucose-6-phosphate is the reactant and that phosphogluconolactone is the immediate product of dehydrogenation (cf. glucose dehydrogenase). Dickens & Glock (139) have shown that glucose-6-phosphate dehydrogenase in animal tissues is TPN-specific, like the yeast enzyme.

6-Phosphogluconic dehydrogenase.—Horecker & Smyrniotis (140) have purified yeast 6-phosphogluconic dehydrogenase, which catalyzes reaction 31:

$$\text{6-phosphogluconic acid} + \text{TPN}^+ \rightleftharpoons \text{ribulose-5-phosphate} + \text{TPNH} + \text{H}^+ + \text{CO}_2 \qquad 31.$$

Since the oxidation and decarboxylation steps have not been separated, it is possible that both may be carried out by a single enzyme. An equilibrium mixture of ribulose-5-phosphate and ribose-5-phosphate is soon obtained, due to the presence of an active ribosephosphate isomerase. The reversibility of the reaction has been demonstrated by three different methods. Preparations from liver and bone marrow behave like the yeast enzyme (cf. 139).

Isocitric dehydrogenase.—Lotspeich & Peters (141) found that pig heart isocitric dehydrogenase was not inhibited by the monosubstituted arsenical compounds, arsenite, and lewisite oxide, but the disubstituted compounds diphenylchlorarsine and some phenarsazines were toxic. Their toxicity could be reversed by BAL or GSH. Cu^{++}, p-chloromercuribenzoate, and iodosobenzoate, but not alkylating agents such as chloracetophenone and iodoacetate, inhibited. It was concluded that isocitric dehydrogenase contains a susceptible -SH group of unusual properties.

Malic dehydrogenase.—Marsh & Militzer (142) have studied some kinetic properties of malic dehydrogenase of a stenothermophilic bacterium, whose optimum temperature is 62–65°C. The activation energy of the reaction with methylene blue (which includes diaphorase) is the unusually low value of 3,700 cal.

Flavoproteins

Cytochrome reductases, which are flavoproteins, have been considered above.

Xanthine oxidase.—Morell (147) has shown that preparations of milk xanthine oxidase contain a flavoprotein, which is rapidly reducible by xanthine or hypoxanthine, as well as flavines which are only very slowly reduced by these compounds. The enzyme activity at different stages of purification is proportional to the concentration of rapidly reducible flavine, not to the total flavine. It is concluded that the active xanthine oxidase is the rapidly reduced flavoprotein, while the slowly reduced flavine compounds are inactivated enzyme. This provides the first strong experimental evidence for the generally accepted belief that xanthine oxidase is a flavoprotein. Xanthopterin and aldehydes also rapidly reduce the flavine to the same extent as xanthine or hypoxanthine, confirming previous evidence that these compounds are also substrates of the enzyme. DPNH, however, produced no measurable reduction showing that the diaphorase activity of xanthine oxidase preparations is not due to the xanthine oxidase but must be ascribed to small amounts of another enzyme. Calcium chloride and other bivalent metal salts are able to detach the FAD[2] of xanthine oxidase from the apoenzyme without precipitating the protein. Xanthine oxidase and other flavoproteins (D-amino acid oxidase and notatin), in the presence of their substrates, reduce free FAD so slowly that free FAD cannot act significantly *in vivo* as a carrier linking these systems with other flavine dehydrogenases. The minimum turnover of xanthine oxidase is 313 $min.^{-1}$ at 19°C. in air.

In opposition to Horecker & Heppel (148) Morell has shown that the reduction of cytochrome-*c* by xanthine and xanthine oxidase initially proceeds as rapidly under anaerobic conditions as in the presence of air; later, the anaerobic reaction is the faster because of competition between oxygen and cytochrome-*c* as hydrogen acceptors. Salts markedly inhibited this reduction. It is not yet known whether xanthine oxidase reacts *in vivo* directly with oxygen or through the cytochrome system.

Klenow (149) has shown that xanthine oxidase preparations oxidize adenine to 2:8-dihydroxyadenine. Since this oxidation is inhibited by 2-amino-4-hydroxy-6-formylpteridine, the very powerful inhibitor of xanthine oxidase, it is concluded that adenine oxidase is probably xanthine oxidase or a similar type of enzyme. This question could be settled by Morell's procedure. The inactivation of xanthine oxidase by ascorbic acid, found by Feigelson (150), is very likely due to H_2O_2 produced during the oxidation of ascorbic acid, since H_2O_2 is known to inactivate this enzyme (151).

Notatin (*glucose oxidase*).—Keilin & Hartree (143) have shown that notatin is almost completely specific for β-glucose. Mannose, altrose, and galactose are oxidized at 1 per cent, 0.16 per cent, and 0.14 per cent of the rate of β-glucose respectively, while allose, gulose, idose, and talose are not oxidized; α-glucose is oxidized at only 0.64 per cent the rate of β-glucose. However, most samples of notatin contain an enzyme (mutarotase) which rapidly catalyzes the interconversion of the two forms so that the equilibrium form of glucose is oxidized to completion. Mutarotase is not identical with notatin and does not arise from notatin during purification. Notatin is a protein of molecular weight 149,000, containing two molecules of FAD per molecule.

Laser (144) has examined the effect of oxygen tension on the activity of notatin and other flavoproteins and on the coupled oxidations catalyzed by these enzymes in the presence of catalase. He has shown that, at low oxygen tensions, catalase (in the absence of ethanol) accelerates the oxidation of glucose by notatin.

L-*amino acid oxidase*.—Burton (145) has shown that this enzyme in *Neurospora crassa* is a typical flavoprotein which catalyzes the reaction:

$$R\cdot CHNH_2\cdot COOH + O_2 \rightarrow R\cdot CO\cdot COOH + NH_3 + H_2O_2. \qquad 32.$$

Like other flavoproteins, ferricyanide and reducible dyes can replace oxygen as hydrogen acceptor. The enzyme preparations contained firmly bound FAD (1 mole per 11,000 g. nondialyzable nitrogen) which was partly reduced by L-alanine; no unphosphorylated riboflavin or the monophosphate were present. This enzyme has also been studied by Thayer & Horowitz (146).

Coenzymes of Oxidizing Enzymes

During the last year, a new coenzyme has been reported and important progress has been made towards the elucidation of the structures and functions of α-lipoic acid and coenzyme A.

Pyridinenucleotides.—Singer & Kearney (152) have found that a new pyridine nucleotide coenzyme, which they name coenzyme III, is required for the oxidation of L-cysteinesulphinic acid to cysteic acid by cell-free extracts of *Proteus vulgaris*. The absorption spectrum of this coenzyme, which was isolated from yeast, is very similar to that of DPN and TPN, and reaction 33 was studied in both directions by the spectrophotometric procedure, commonly used with dehydrogenases reacting with these coenzymes.

$$\text{L-cysteinesulphinic acid} + Co^{+} \rightleftharpoons \text{cysteic acid} + Co.H + H^{+} \quad 33.$$

(Co = new coenzyme). From the solubility of the heavy metal salts, chromatographic behaviour, stability, and inactivation by nucleotide pyrophosphatase, Singer & Kearney suggest the structure to be nicotinamide-ribose-5-pyrophosphate.

Burton (153) has remeasured the oxidation-reduction potential of DPN. The value calculated both from earlier data on the ethanol-DPN-acetaldehyde equilibrium and new data on the isopropanol-DPN-acetone equilibrium (catalyzed by yeast alcohol dehydrogenase) is -0.320 ± 0.005 volts at pH 7.0, 25°C. This is considerably different from the previously generally accepted value (-0.28 volt).

Colowick *et al.* (154) have found that desamino-DPN has the same activity as DPN in some enzyme systems (e. g., liver alcohol dehydrogenase) and is less active in others; desamino-DPNH is more active than DPNH for pyruvate reduction by lactic dehydrogenase.

Coenzyme A.—Lipmann *et al.* (155) have shown that the sulphur group of CoA is in the form of β-mercaptoethylamine linked through its amino group to the carboxyl group of pantothenic acid, as in pantotheine, the growth factor for *Lactobacillus bulgaricus.* After removing contaminating disulphides, a preparation 90 to 93 per cent pure was obtained; per mole of pantothenic acid this contained adenine, 1.05; total P, 2.83; monoester P, 0.96; sulphur, 1.07 moles.

Baddiley & Thain (156) have described the synthesis of pantotheine and of the 2′- and 4′- monophosphates and the 2′, 4′-cyclic monophosphate of pantothenic acid. In the products of the acid and alkaline hydrolysis, they have identified the 4′-monophosphate, the 2′, 4′-cyclic monophosphate and adenosine-5′-phosphate and have proposed for CoA the formula:

$$R \cdot O \cdot POOH \cdot O \cdot POOH \cdot O \cdot CH_2 \cdot CMe_2 \cdot CHOH \cdot CONH \cdot (CH_2)_2 \cdot CONH \cdot (CH_2)_2 \cdot SH$$

where R is adenosine containing an additional phosphate group. The points of attachment of the pantothenic acid moiety and of the additional phosphate group to the ribose of the adenosine are not certain; the possibilities suggested by Baddiley & Thain are 5′ and 2′ or 3′ respectively. Kaplan *et al.* (157) have shown, by means of a nucleotidase from barley which is specific for "b" nucleotides, that the monoester phosphate group is in the "b" form, as contrasted to TPN, which is an adenylic acid "a" derivative. Baddiley & Thain also synthesized derivatives of 2-acetylthioethylamine and found that they rapidly acetylated hydroxylamine by a nonenzymic reaction, which supports the finding of Lynen & Reichert (9) that CoA brings about acetylation by acetyl transfer through its thiol group.

Lipmann *et al.* (5, 158) have found that the formation of acetyl-CoA from acetate, CoA, and ATP by yeast and pigeon liver extracts involves AMP and inorganic pyrophosphate, and they suggest the intermediate formation of CoA-pyrophosphate:

$$\text{ATP} + \text{CoA} \rightleftharpoons \text{AMP} + \text{CoA-pyrophosphate} \quad 34.$$

$$\text{CoA-pyrophosphate} + \text{acetate} \rightleftharpoons \text{acetyl-CoA} + \text{pyrophosphate} \quad 35.$$

CoA-pyrophosphate has not yet been identified directly. Peters *et al.* (159) have established that the inhibitory effect of fluoracetate on respiration is due to the formation of fluorocitrate, which is a competitive inhibitor of aconitase. If reactions 34 and 35 represent the only mechanism of acetate activation, *in vivo*, fluoroacetate must enter reaction 35 in place of acetate to form fluoracetyl-CoA which condenses with oxaloacetate to form fluorocitrate.

Beinert *et al.* (160) have described a method of preparing CoA from yeast, and Wilson (161) has described the preparation of acetyl-CoA by acetylation of CoA with thiolacetate.

α-Lipoic acid (*pyruvate oxidation factor*).—This compound which has now been synthesized is an intramolecular disulphide of a dimercapto-n-octanoic acid, unsubstituted in the α and β-positions (162). Gunsalus (16a) has suggested that it is the dithiol grouping earlier postulated by Stocken & Thompson (163), on the basis of inhibition by lewisite and reversal by the dithiol BAL,[3] that is required for the oxidation of pyruvate. Lipothiamide has been discussed on p. 20.

Flavine compounds.—Whitby (165) has shown that a new flavine compound, riboflavinyl glucoside, is formed by a transglycosidation of D-glucose from maltose or glycogen to riboflavin, in the presence of an enzyme in liver. Its biological function is not known.

Catalase and Peroxidase

George & Irvine (166) found by direct titration that peroxidase-complex II is not a peroxide of peroxidase with two oxidation equivalents in excess of that of peroxidase but contains only one excess oxidation equivalent. George therefore proposed the mechanism[5]

$$\text{peroxidase} + H_2O_2 \rightarrow \text{compound I} \xrightarrow[\text{reduction}]{\text{one equiv.}} \text{compound II} \xrightarrow[\text{reduction}]{\text{one equiv.}} \text{peroxidase.} \quad 36.$$

This mechanism is supported by Chance (167) who found, by direct titration, that the transformation of complex I to complex II involved one equivalent reduction. George's mechanism explains the puzzling feature in Chance's earlier work that the transformation of complex I to complex II was greatly accelerated by the presence of a hydrogen donor.

More recently, George (168) has obtained qualitative evidence for similar structures of the analagous complexes of catalase. The transformation of compound I to compound II is speeded by ferrocyanide which does not, however, reduce compound II of catalase to the free enzyme. But compound II can be reduced to free enzyme by $Na_2S_2O_4$, NH_2OH, N_2H_4, nitrite, as-

[5] George prefers to call the intermediates, compound I and II respectively instead of complex I and II, which are the terms used by Chance. The two expressions will be used interchangeably in this review.

corbic acid, and many phenols. The phenols also speed the transformation of compound I to II. Chance (170) postulated that complex I is directly reduced to the free enzyme either by H_2O_2, ethanol, or nitrite during the enzymic activity of catalase. Since nitrite reduces complex II to the free enzyme, George suggests that the reaction between complex I and nitrite may occur in steps, as with peroxidase. He also suggests that the same mechanism might be operating in the oxidation of alcohol by H_2O_2 and catalase, only in this case the reaction between compound II and the one-equivalent oxidized alcohol molecule would be so fast that the steady-state concentration of compound II would be very low.

Complex II was first observed by the action of ascorbic acid on catalase by Lemberg & Foulkes (169), who incorrectly believed that the compound was one between ascorbic acid and catalase. Chance (170) and Keilin & Hartree (171) found that it was the H_2O_2 formed by the autoxidation of ascorbic acid which was responsible for the complex. Nevertheless, it now seems from George's work that the ascorbic acid plays two roles: (*a*) its autoxidation provides the H_2O_2 and (*b*) it promotes the reduction of complex I to complex II. When H_2O_2 is provided by notatin-glucose, as in the experiments of Chance and Keilin & Hartree, the transformation of complex I to complex II depends upon a spontaneous reaction, apparently with reducing substances in the preparations (172).

Tauber & Petit (173) have simplified the method of obtaining crystalline catalase from cow liver. Brown (173a) has demonstrated by a solubility test technique that crystalline ox-liver catalase (*Kat.f.* = 72,000) contains two distinct catalases with *Kat.f.* values of 45,000 and 180,000.

Beers & Sizer (174) measure catalase activity by following the disappearance of H_2O_2 spectrophotometrically at 240 mμ. The velocity of the reaction increased only slightly with temperature. This corresponds to an activation energy of 600 calories; the two reactions involved have about the same activation energy.

A constitutional abnormality important from the point of view of the physiological function of catalase has been found in man by Nakamura *et al.* (175). Although the subjects described contain no catalase in the blood, liver, or muscle, they show no signs of metabolic disturbance, except for a marked susceptibility to a severe dental infection. The first clear-cut evidence that the reaction of catalase with H_2O_2 occurs in the intact cell has been obtained by Chance (176), who identified catalase-peroxide complex I in respiring suspensions of *Micrococcus lysodeikticus*. The complex was decomposed by formate or nitrite, shown as hydrogen donors for the complex, and also when the suspension became anaerobic due to the exhaustion of oxygen from the suspension by respiration of the organism. Chance therefore suggests that endogenous donor is present.

Knox (177) has shown that tryptophan peroxidase is inhibited by carbon monoxide (light-sensitive) and suggests a ferric-ferro change during the action of this enzyme which differs from other peroxidases in that the per-

oxide required is generated in some unknown way in one step of the reaction. These properities recall the oxidation of dihydroxymaleic acid by horse-radish peroxidase [see Chance (178)]. Goldacre (178a) has shown that the oxidation of indole-3-acetic acid by peas involves H_2O_2 and a peroxidase; the source of the H_2O_2 is not known (cf. 61).

Michaelis Constants and Dissociation Constants

Several papers dealing with the relation between Michaelis constants and dissociation constants have appeared during the last year. It is to be hoped that they will be instrumental in stopping the widespread habit of equating the Michaelis constant (the substrate concentration which gives half the maximum velocity) with the dissociation constant of the enzyme-substrate compound. Over 25 years ago, Briggs & Haldane (179) showed that for the reaction sequence $E+S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES$; $ES \xrightarrow{k_3} E+\text{products}$ (E = enzyme, S = substrate, ES = Michaelis enzyme-substrate compound), K_m (Michaelis constant) $=(k_2+k_3)/k_1$. Only when k_3 is small compared with k_2, as assumed by Michaelis & Menten (180) will $K_m=k_2/k_1=K_D$ (dissociation constant of the enzyme-substrate compound). Chance's measurements (181) of the actual rate constants of horse-radish peroxidase provided the first quantitative evidence in support of the essential features of the Michaelis & Menten theory. At the same time they showed that it was the Briggs-Haldane modification which was applicable, since k_2 was very small compared with k_3. It must be mentioned, however, that the more recent studies by Chance and by George have shown that peroxidase kinetics are particularly complicated and that two intermediate compounds are involved. Chance & Smith (1) overcome this difficulty by defining the Michaelis complex as that intermediate whose concentration determines the overall reaction velocity. George (166), on the other hand, considers that the secondary complexes of peroxidase and H_2O_2 should no longer be regarded as Michaelis-Menten enzyme-substrate complexes, since the substrate molecule is not a component part of the complex, but as reaction intermediates in the same sense that free radicals and Michaelis semiquinones are reaction intermediates.

Succinic dehydrogenase may perhaps be considered more typical of oxidizing enzymes in general than peroxidase. It is of interest, therefore, that Slater & Bonner (36) have shown that, in the case of this enzyme also, k_2 (2 sec.$^{-1}$) is relatively small compared with k_3 (32 sec.$^{-1}$). This means that K_D (3×10^{-6} M) is much smaller than K_m (4.8×10^{-4} M). Laser's (144) finding that the K_m (glucose) of notatin (glucose oxidase) depends upon oxygen tension strongly suggests that, with this enzyme also, k_2 is not large compared with k_3. Ogura (182) has also concluded that the dissociation constant of the enzyme-substrate compound of the glucose dehydrogenase of *Aspergillus oryzae* is constant, although the K_m varies with the hydrogen acceptor used. Smith *et al.* (182a) believe that $K_m=k_3/k_1$ in the case of the hydrolytic

enzyme, carboxypeptidase; substitution of D_2O for water affected both K_m and k_3 equally.

The recent studies of Theorell, Bonnichsen & Chance (131) have shown that the lack of correspondence of K_m with K_D also applies to DPN-dehydrogenase complexes, e.g., K_m of DPNH for reaction 37

$$\text{DPNH} + \text{CH}_3\text{CHO} + \text{H}^+ \rightarrow \text{DPN}^+ + \text{CH}_3\text{CH}_2\text{OH} \qquad 37.$$

catalyzed by liver alcohol dehydrogenase, is 200 times K_D at pH 7. This is not surprising, if the full reactions involved are considered viz.,

$$\text{E} + \text{DPNH} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{E.DPNH} \qquad 38.$$

$$\text{E.DPNH} + \text{CH}_3\text{CHO} + \text{H}^+ \overset{k_3}{\rightarrow} \text{E.DPN}^+ + \text{CH}_3\text{CH}_2\text{OH} \qquad 39.$$

$$\text{E.DPN}^+ \underset{k_5}{\overset{k_4}{\rightleftharpoons}} \text{E} + \text{DPN}^+ \qquad 40.$$

The solution of the steady-state kinetic equations[6] by Theorell & Chance (131) leads to a complicated expression for K_m of these reactions, which can, under certain limiting conditions, be simplified to the expression $K_m = k_4/k_1$, which equals K_D $(= k_2/k_1)$ only when $k_2 = k_4$, which is certainly not the case with this enzyme. These equations show, too, that DPN can act as a competitive inhibitor of reaction 37 (aside from any reversal of the reaction) which should be kept in mind when reactions of this type are used to measure dehydrogenase activity. Bücher & Garbade's (132a) finding that the K_m(DPN) of glyceraldehydephosphate dehydrogenase depends upon the concentration of arsenate is readily explicable on the basis of these equations.

Theorell & Bonnichsen (131) have calculated that since the dissociation constant of the DPN-alcohol dehydrogenase is much greater than that of the DPN-enzyme complex, the E'_0 of the bound DPNH-DPN system is greater than that in solution [cf. Dixon (183)]. At pH 7.0 the respective values are -0.208 and -0.275 volt.

Schwert & Hakala (184) have analyzed the kinetics of lactic dehydrogenase on the basis of a ternary complex in equilibrium with substrate, enzyme, and coenzyme. The above studies on alcohol dehydrogenase make it very doubtful that this is a profitable approach. No valid experimental evidence has yet been obtained for the existence of any ternary complex in enzyme reactions, despite the popularity of such complexes in many theortical formulations. The bimolecular reaction of acetaldehyde with the enzyme-DPNH complex (reaction 36) is consistent with the existence of a Michaelis constant for acetaldehyde. It must be emphasized that the existence of a Michaelis constant does not necessarily imply a Michaelis complex (cf. 58).

[6] Unpublished work of the reviewer in 1950 confirms the solution of Theorell & Chance.

The low dissociation constant of alcohol dehydrogenase and DPNH indicates a firm binding which Theorell & Bonnichsen believe involves -SH group. The considerable change of spectrum also shows that a definite chemical bonding is involved. Morell (147) has shown that the combination of FAD with xanthine oxidase apoenzyme also causes a very marked change in the absorption spectrum; the absorption at 450 mμ is doubled and considerable absorption in the green appears. Singer & Pensky (185) have observed a slight increase in the absorption below 290 mμ when diphosphothiamine combines with the apoprotein of the α-carboxylase of wheat germ in the presence of Mg^{++} and a further increase on the addition of acetaldehyde.

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PROTEOLYTIC ENZYMES[1,2]

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INTRODUCTION

The treatment of the present subject follows essentially the principles upon which a previous review by one of the authors was based. We should perhaps point out that since other review series have appeared in later years (e.g., *Annual Review of Physiology* and *Annual Review of Microbiology*) we have limited our treatment of medical, physiological, and microbiological enzyme chemistry. For this reason a great number of investigations on the enzymes of blood coagulation and fibrinolysis have been omitted in the hope that they will be discussed elsewhere.

CHEMISTRY

Preparative and Constitutional Chemistry

Pepsin.—In an extensive study of the heat of denaturation of pepsin Buzzell & Sturtevant (1) have found that pepsin, in the same pH region in which it loses its proteolytic activity, undergoes an endothermic reaction. ΔH of this reaction is dependent upon pH, showing a pronounced maximum around pH 6.4 at 35°C. and a smaller one around pH 7.2 at 15°C. At 35°C. the enthalpy change rises from about 20 kcal./mole at pH 6.1 to about 50 kcal./mole at pH 6.4, and falls again to 25 kcal./mole at pH 6.5. As the authors rightly point out, it is difficult to find an entirely convincing explanation for this phenomenon, mainly because of the surprising magnitude of the effect. Since the experiments were carried out in phosphate buffer there can be no doubt that reactions of the following type must play a role:

$$RCOOH + HPO_4^{=} \rightleftarrows RCOO^- + H_2PO_4^-$$

(ΔH from left to right ca. −2 kcal.)

$$>NH + H_2PO_4^- \rightleftarrows >NH_2^+ + HPO_4^{=}$$

where >NH signifies histidine; (ΔH from left to right ca. −5 kcal.)

They involve the only groups in pepsin that are supposed to ionize in the pH region studied. However, when we come to the question of creating a sufficient number of such groups in the process of denaturation, e.g., by liberation from ionic bonds [see Zaiser & Steinhardt (2)], we meet with the difficulty that the pepsin molecule contains only two histidine residues, corresponding to a maximum variation of ΔH per pepsin molecule of only 6 kcal. in the pH region 6 to 6.4. These ionization effects must therefore be "amplified" in some way, which means that other properties than the ionization pattern

[1] The survey of the literature pertaining to this review was concluded November 1, 1952.

[2] The following abbreviation is used: DFP for diisopropyl fluorophosphate.

must vary with pH. A noninstantaneous heat absorption might therefore be anticipated if protein already denatured at pH 6 were brought to pH 6.4.

As regards the possible connection of the heat absorbing process with that responsible for the loss in enzymatic activity of pepsin, the authors emphasize the point that the rate curves for the loss in activity do not coincide with those of the endothermic process, the latter being of the first order, the former corresponding more closely to second order curves. In addition the dependence of the rate upon pH is strikingly different. It is therefore unlikely that the endothermic process is intimately connected with the groups directly involved in the catalytic activity.

In connection with the above discussed paper an investigation by Casey & Laidler (3) on the heat inactivation of pepsin may be mentioned. The main result of these authors, viz., that the order of the reaction increases with decreasing initial concentration of pepsin at pH 4.83 and 57.4°C. (acetate buffer), could not be reproduced by Buzzell & Sturtevant at lower temperature and higher pH (phosphate buffer). This may again be taken as an indication that the course of the process of denaturation may vary strongly with the conditions under which it is studied. It should, however, be emphasized that traces of impurities from the water or from the glass of the vessels may become important in extremely dilute protein systems (molarities of pepsin down to 10^{-6}). One cannot entirely exclude the possibility that a trace of a stabilizing substance may play a role in the findings of Casey & Laidler.

Cumper & Alexander (4) have studied the rigidity of adsorbed films of pepsin at an air-water interface under different conditions (temperature, pH, and ionic strength). Their results are compatible with the general view that the adsorbed molecules undergo a surface denaturation and are stepwise incorporated in the closely packed surface layer of unfolded peptide chains.

Williamson & Passmann (5) have reported experiments on the chemical structure of pepsin. By means of Sanger's method they have reached the conclusion that the pepsin molecule consists of two peptide chains linked together by S—S bonds. One of these chains is open and has leucine as the N-terminal amino acid. The other is apparently closed. No detailed data are as yet available.

Lundquist & Seedorff (6) [see also Lundquist (7)] have reported the presence of pepsinogen in human seminal fluid. The activated enzyme could be crystallized. Crystal shape and specificity matched those of ordinary pepsin within the limits of the investigation.

Rennin.—Schwander, Zahler & Nitschmann (8) have carried out a physical and chemical investigation of crystalline rennin. The molecular weight was found to be 40,000 ($s_{20,w}=4S$; $D_{20}=9.5\times10^{-7}$ cm.^{2}/sec.; $V_{20}=0.749$). The preparation was not quite homogeneous in the ultracentrifuge, but no inhomogeneity was found by paper electrophoresis (isoelectric point about pH 4.5). The amino acid pattern is characterized by being rather low in tyrosine and sulphur-containing amino acids. Berridge (9) has studied the

stability of different rennin preparations and found that the major component decomposes in a first order reaction. The experiments are preliminary.

Trypsin.—The amphoteric properties of trypsin were studied by Duke, Bier & Nord (10, 11) who found per 10,000 gm. of trypsin, six carboxyl groups, dissociating in the pH region 2 to 6. Addition of calcium ions caused a shift of the ionization curve to a lower pH, a fact which the authors correlate with the stabilizing effect of calcium upon trypsin. Since from a previous work [Bier & Nord (12)] it appeared likely that all the nonpeptide-bound carboxylic groups of aspartic and glutamic acids are present as amides, the above named six free carboxylic groups evidently have to be terminal ones (unless ammonia has been transferred from the dicarboxylic amides to the terminal carboxylic groups, which is unlikely). The course of the titration curves, however, does not indicate the high acidity (pK 3.3) normally assigned to such carboxylic groups in isolated peptide chains, nor is it compatible with the assumption of an unusually close packing of carboxylic groups in the protein molecule which would cause a broadening of the ionization region (13). The question therefore needs further investigation.

Bier & Nord (14) have studied the effects of high intensity electron bombardment on dissolved trypsin. The observed loss of activity increased with increasing acidity. No stabilization by calcium ions was found.

As reported by Bowman (15) trypsin may be treated with hypochlorite without loss of enzymatic activity; the chemical change induced is not discussed.

Although it is uncertain how to classify the protein involved, the interesting fiber formation in pancreatic extracts and trypsinogen solutions should be mentioned here [Gross (16, 17, 18)]. The conditions under which this phenomenon occurs are evidently different from those which cause insulin to form fibrils.

During the past year a great many investigations have been carried out on the combination of trypsin with the inhibitor diisopropyl fluorophosphate (DFP) and related compounds. Jansen & Balls (19) report the crystallization of inactive DFP[2]-trypsin containing approximately one mole of phosphorus per mole trypsin ($M=20,700$). Neurath *et al.* (20, 21) have found that the phosphorus content varies somewhat from preparation to preparation. The lowest values found correspond to that of Jansen & Balls. DFP-trypsin does not react with pancreatic or soybean inhibitors like crystalline trypsin. It sediments in the ultracentrifuge as a single molecular species over a wide pH range (sedimentation constant at infinite dilution, $s_{20,w}=2.5\ S$) in contradistinction to active trypsin which forms aggregates at finite concentration but tends towards the same value of $s_{20,w}$ (which corresponds to a molecular weight of about 24,000).

Kilby & Youatt (22) have studied the reaction of trypsin with diethyl-*p*-nitrophenyl phosphate (E 600) and O,S-diethyl-O-*p*-nitrophenyl thiophosphate. In the reaction, which is of first order, *p*-nitrophenol is liberated in an amount corresponding to two moles per mole of trypsin if the molecular

weight of the enzyme is taken as 34,900. On the basis of Neurath's estimated value for the molecular weight it would be closer to one mole/mole.

As regards specific biological inhibitors of protein nature Dobry & Sturtevant (23) have studied the heat of reaction between trypsin and soybean inhibitor and found it to be -2.4 ± 0.6 kcal./mole in the pH region 2.7 to 4.0. McLaren (24) has investigated the volume change accompanying the same reaction and found a value of $+25$ ml./mole at pH 6. A reasonable explanation for this phenomenon is that some pairs of oppositely charged ions are brought to a greater nearness in the process, whereby electrostriction decreases. If two monovalent ions are brought from the distance a to the distance b (Å) in water the heat of reaction is about $-4(a-b)/ab$ kcal., and the value found by Dobry & Sturtevant may therefore also be explained qualitatively on this electrostatic basis, especially since the reaction is hardly complete in the pH-range studied by them. That serum albumin imitates trypsin in combining with the inhibitor under heat evolution seems to us of little weight as an argument against the specific nature of the enzyme-inhibitor reaction. Everything depends here upon the spatial position of the active group of trypsin relative to the region of contact between the two proteins.

Mishuck & Eirich (25) have studied the combination of trypsin and soybean inhibitor in surface films. Force-area curves suggest that the reaction proceeds between dimers of the two proteins.

Fraenkel-Conrat *et al.* (26) have isolated and characterized a trypsin inhibitor from lima beans. The purified (not crystallized) inhibitor is of high sulphur and cystine content (5.5 and 16 per cent respectively). Extensive reduction of the disulphide bonds resulted in loss of activity and so did blocking of part of the amino groups. Several other chemical changes, e.g., esterification of COOH groups, were without effect. The inhibitor seems to be markedly different from the soybean inhibitor.

Laskowski, Mars & Laskowski (27) have compared a crystalline trypsin inhibitor from colostrum with other known inhibitors and found marked differences. One μg. of trypsin was inactivated by 1 μg. of soybean-, 0.5 μg. of pancreatic,- 0.43 μg. of colostrum-, and 0.39 μg. of Kazal's inhibitor (28). The isoelectric points were: colostrum inhibitor, pH 4.2; pancreatic inhibitor, pH 8.7. The molecular weight of the colostrum inhibitor-trypsin complex was unexpectedly high (89,000).

Bier and co-workers (29) have fractionated ovomucoid by electrophoresis and demonstrated five components of which at least three, constituting 92 per cent of the preparation investigated, are active inhibitors for trypsin. Their isoelectric points are at pH 4.41, 4.28, and 4.17.

Gorini & Audrain (30, 31) have studied the effect of Ca^{++} upon ovomucoid and its complex with trypsin. A definite but small proteolytic activity could be ascribed to the trypsin-ovomucoid complex.

Chymotrypsin.—The isoelectric point of chymotrypsinogen has been determined by Ingram (32), using the Donnan equilibrium method. The value obtained, viz, pH 6 to 6.5, deviates strongly from previous figures (pH 9.1 to

9.5) found by electrophoresis. We find this deviation worth investigating but cannot follow the author when he emphasizes the superiority of the method over other methods currently in use. The measurement of small pH differences between a dilute buffer and a 10 per cent protein solution has disadvantages of its own (protein error of the electrode, diffusion potentials.)

McLaren & Waldt (33) have studied the ultraviolet absorption spectrum of α-chymotrypsin and compared it with that of a mixture of amino acids having the same residual make-up as the protein. At the maximum, 280 mμ, the contribution of the peptide bonds to the absorption is below 3 per cent, while at 240 mμ it is considerable.

Doherty (34) has investigated the inactivation of α-chymotrypsin by gamma rays in dilute solutions. The fall in activity is not accompanied by changes in substrate binding capacity (Michaelis constant). Specific inhibitors, as well as substrates, protect the enzyme.

Weil & Buchert (35) have reported experiments in which α-chymotrypsin was photooxidized by visible light in the presence of traces of methylene blue. A parallel decrease of both proteinase and esterase activities was observed at pH 7.4. Complete inactivation was reached at an oxygen uptake of eight atoms per mole of enzyme. Histidine and tryptophan are the centers of the oxidative attack.

The N-terminal groups of chymotrypsinogen and α-chymotrypsin have been determined by Desnuelle, Rovery & Fabre (36, 37, 38). The zymogen is either a peptide ring, or the terminal groups are bound in some unknown way. α-Chymotrypsin (inhibited by DFP[2], see below) has two terminal amino groups belonging to alanine and isoleucine. Gladner & Neurath (39) have determined the C-terminal amino acids in the same proteins by means of carboxypeptidase. Chymotrypsinogen seems again to be devoid of terminal groups while leucine and tyrosine were rapidly released from α-chymotrypsin (inhibited by DFP, see below). The enzyme therefore seems to contain two open peptide chains.

An interesting observation is reported by Stahmann & Becker (40) who have reacted chymotrypsin with N-carboxyglycine anhydride at pH 7.4 and obtained a glycine-enriched chymotrypsin (about 90 moles of glycine per mole instead of the normal 25) without loss in enzymatic activity. The product was apparently homogeneous and gave cross-reaction with antiserum against chymotrypsin. Since a marked difference in the titration curves of the glycine-enriched and the original protein was observed in the region pH 7 to 11, it is reasonable to assume that polyglycine molecules have become attached to the nonterminal amino groups (lysine) by means of peptide bonds.

Smith & Brown (41) have reported that the anomalous sedimentation (tendency to association) shown by chymotrypsin is found also in DFP-chymotrypsin, and hence there seems to exist a characteristic difference between trypsin and chymotrypsin in this respect. However, oxidized chymotrypsin (42), inhibited by DFP, sediments as a monomer at pH 4 ($M = 22{,}500$).

Jansen & Balls (43) have studied the combination of β- and γ-chymotrypsin with DFP[2]. The inactive, crystalline, phosphorus-containing compounds obtained showed combining weights of 24,200 and 23,900 respectively (weight per P atom).

Shaffer, May & Summerson (44) have reported that DFP-chymotrypsin prepared with phosphorus-labelled DFP, upon partial hydrolysis by enzymes and acid, yields serine phosphate in appreciable quantities (yield 30 per cent). The authors cautiously conclude that the ultimate, but not necessarily the immediate, site of attachment of DFP to chymotrypsin is at an OH-group of serine. In this connection an investigation by Ashbolt & Rydon (45) may be mentioned in which the ready reaction of DFP with the phenolic hydroxyl of tyrosine is reported. A transfer of the diisopropyl phosphate group from tyrosine to serine during the mild hydrolysis may not be out of question.

Hartley & Kilby (46) have investigated the reaction of diethyl-*p*-nitrophenylphosphate (E 600) with α-chymotrypsin [see Kilby & Youatt (22)]. One mole of nitrophenol is liberated per mole of enzyme, and hence the results support the hypothesis of a single active centre in chymotrypsin.

Other enzymes.—Merten *et al.* (47) have carried out a preparative investigation of the enzymes in acid extracts of the stomach mucosa of hog. By salt precipitations and electrophoresis it was possible to distinguish a "catheptic" component, with pH optimum at 3.5, from the pepsin component. The latter could be prepared in a pure state, i.e., without catheptic activity, whereas the catheptic component always showed some peptic activity. The enzymes are apparently closely related, and it seems hardly justified to characterize one of them as a cathepsin on the present experimental basis.

Tallan, Jones & Fruton (48) have accomplished an appreciable purification of cathepsin C of beef spleen (fractionation with ammonium sulphate and precipitations by ethanol and zinc ions at low temperature). The enzyme resembles chymotrypsin in its specificity.

An electrophoretic study of a streptococcal proteinase and its precursor has been made by Shedlovsky & Elliott (49). Both substances have been crystallized and characterized by Elliott (50, 51). Their isoelectric points are at pH 8.42 and 7.35 respectively.

Robinson, Birnbaum & Greenstein (52) have used the butanol method of Morton (53, 54) for the solubilization of L- and D-peptidases of hog kidney particulates. In certain cases a purification of more than 600-fold was achieved.

Prolidase has been prepared by Adams & Smith (55, 56, 57) from erythrocytes (100 to 400-fold purification). The pH optimum was 7.8 (glycylproline) and activation by Mn^{++} was observed.

Güntelberg & Ottesen (58, 59) have crystallized a proteolytic enzyme secreted by ***Bacillus subtilis***. The enzyme protein appears to be homogeneous both in electrophoresis and sedimentation experiments. The isoelectric point was found to be at pH 9.3, and the sedimentation constant, 2.7 S. Fukumoto & Negoro (60, 61) have studied the conditions for the formation of proteinase

by *Bacillus amyloliquefaciens*. They have succeeded in crystallizing the enzyme from aqueous acetone. Lepow, Katz & Pensky (62) and Ogle (63) have purified a proteinase from *Clostridium histolyticum* by precipitation in aqueous alcohol [addition of Zn^{++}, Ogle (63)].

Chemistry of Action

Specificity and kinetics.—Baker (64) has prepared new substrates for pepsin of the type, CH_3—CONH—C(R_1)H—CONH—C(R_2)H—COOH, where R_1 and R_2 are L-tyrosyl, L-diiodotyrosyl or L-phenylalanyl side chains. They are rapidly split in strongly acid solution (pH 2) where the carboxylic group is uncharged. Replacement of the L-forms by D-forms gives compounds that are resistant to pepsin.

Botvinik & Avaeva (65) report hydrolysis by pancreatin and crystalline trypsin of an ester bond between N-acylated phenylalanine and the hydroxyl group of N-acylated serine. No splitting occurred if the carboxylic group of the serine moiety was blocked by esterification. In view of the fact that N-acylated phenylalanine esters are typical substrates for chymotrypsin, the equivalence of pancreatin and trypsin as catalysts is hard to understand.

Niemann and co-workers (66 to 71) have continued their investigations of the kinetics of the hydrolysis of different substrates by α-chymotrypsin, e.g., acetyl-L-tryptophan amide, acetyl-L-tyrosine amide, nicotinyl-L-tyrosine amide, acetyl-L-phenylalanine amide, and hippuric acid methylester. The latter was found to be split rapidly by α-chymotrypsin. As for details of these interesting investigations we must refer to the original papers.

Hartley & Kilby (46) have made the observation that *p*-nitrophenyl ethyl carbonate and *p*-nitrophenyl acetate are substrates for chymotrypsin. The turnover numbers are, however, very small.

Mycek, Elliott & Fruton (72) have studied the specificity of the crystalline streptococcal proteinase mentioned on p. 62 (49). The enzyme was shown to attack at peptide bonds involving the carboxyl group of an L-arginine, L-lysine, L-glutamic acid, L-aspartic acid, or L-histidine residue. The presence of a glycyl residue in the substrate appeared to block the action on adjacent sensitive peptide bonds. A similar investigation of a proteinase from *Clostridium histolyticum* has been made by Ogle (63). In its activated state [activators: cysteine, glutathione; compare Lepow, Katz & Pensky (62)] this enzyme is able to hydrolyze substrates like L-arginine methylester and α-N-benzoyl-L-arginine amide (or ester). L-Lysine methylester was split very slowly. Other substrates (for α-chymotrypsin or papain) were not attacked.

Pechmann (73) has investigated the specificity and inhibition of a purified proteinase from "Dymal," a commercial product prepared from the *Aspergillus flavus-oryzae* group. A similar preparation was used by Wallenfels (74) who also studied the hydrolysis of casein and hemoglobin by the crystalline *Aspergillus oryzae* enzyme of Crewther & Lennox (75).

Smith, Lumry & Polglase (76) have studied the effect upon carboxy-

peptidase of a number of carboxylic acids which combine with the enzyme through the side chain and the free carboxylic group and cause inhibition. The combining forces appear to be short range, weak, intermolecular forces of the van der Waals type. Further investigations have been made by Lumry, Smith & Glantz (77) on the effect of various extrinsic factors on the kinetic parameters of carboxypeptidase action. The substrates studied were especially carbobenzoxy-glycyl-L-tryptophan-L-phenylalanine and carbobenzoxy-glycyl-L-tryptophan-L-leucine. The effects of deuterium oxide, of the ionic strength, and of specific inhibitors were investigated. The paper contains a discussion of the significance of K_m, the Michaelis constant, and arguments in favor of regarding it as a dynamic constant:

$$K_m = (K_{-1} + K_0)/K_1.$$

The velocity constants involved appear in the reaction scheme:

$$E + S \underset{K_1}{\overset{K_1}{\rightleftarrows}} ES \xrightarrow{K_0} P + E$$

(E, S, and P are enzyme, substrate, and reaction product, respectively). In this connection we may refer to papers by Christiansen (78), Darling (79), Schønheyder *et al.* (80) and Segal, Kachmar & Boyer (81) in which a more detailed analysis of such systems is attempted.

Peptidase action on peptides containing proline and hydroxyproline has been studied by Davis & Adams (82) [see also Neuman & Smith (83)]. Mn^{++} and Cd^{++} activate the splitting by kidney prolinase of L-prolylglycine and similar dipeptides containing L-proline and hydroxy-L-proline. These ions also activate the hydrolysis of substrates like L-proline amide and hydroxy-L-prolylglycylglycine. Erythrocyte prolidase is activated by Mn^{++} but inhibited by Cd^{++} and appears to be a dipeptidase specific for substrates of the type glycyl-L-proline.

Birnbaum and co-workers (84) and Rao *et al.* (85) have investigated the specificity of amino acid acylases acting on substrates of the type R_1CH_2CO—$NHCHR_2COOH$, where R_1 is mostly H or Cl while R_2 has been varied extensively. There appears to be two distinct acylases in homogenates of hog kidney, one with a very broad range of specificity, the other, aspartic acid acylase, acting only on chloroacetyl- and acetyl-aspartic acid.

Specific inhibitors and activators.—Mattenheimer, Nitschmann & Zahler (86) have found that pure crystalline rennin does not act as a phosphatase. However, upon addition of a thermostable activator from crude rennin or milk it acquires activity toward phosphopeptone from casein.

Gawron & Cheslock (87) have observed an activation of papain by arylamides of mercaptoacetic acid. They propose an activation according to the scheme

$$\text{enzyme-S—SR}_X + \text{HSR}_A \rightarrow \text{enzyme-SH} + \text{R}_X\text{—S—S—R}_A$$

where R_ASH is the activator and R_xSH a low molecular weight substance forming a mixed disulphide with the enzyme when in its inactive state. Ap-

parently no attempts have been made to isolate the compound R_x—S—S—R_A(whereby R_x could be identified).

Huang & Niemann (88) have shown that chloramphenicol (chloromycetin) acts as a competitive inhibitor in the system α-chymotrypsin-nicotinyl-L-tryptophan amide. Acetyl-D-tryptophan amide and acetyl-D-tryptophan methylester act as competitive inhibitors in the milk clotting by α-chymotrypsin (89). The enzyme-inhibitor dissociation constants of α-chymotrypsin and competitive inhibitors containing D-tryptophan have been determined (90).

Green *et al.* (91) and Neurath *et al.* (92) report that certain divalent cations (Ca^{++}, Mn^{++}, Cd^{++}, Co^{++}) which stabilize trypsin also increase the enzymatic activity of trypsin and chymotrypsin (substrates: benzoyl-L-arginine ethylester and acetyl-L-tyrosine ester, respectively). Zn^{++} inhibits these enzymes.

According to Bresler & Rozentsveig (93) commercial trypsin and trypsin in pancreatic juice contain one atom of Cr^{++} per mole of protein. By dialysis inactivation occurs, but reactivation can be effected with either Cr^{++} or Mg^{++}. In crystalline trypsin Cr^{++} is replaced by Mg^{++}. Mg^{++} is the active metal in chymotrypsin. We refer the readers to papers by Smith [e.g. (94)] who has recently argued in favor of enzymes being metalloproteins.

Breakdown of proteins.—The problem of the initial stages of the breakdown of proteins has been treated by several authors. By means of the ultracentrifuge, Williams *et al.* (95) have studied the path of the hydrolysis of γ-globulins by pepsin. The paper is theoretically and experimentally important [see also Gosting (96)], but it seems to us that the system is too complicated to permit definite conclusions. An investigation of a simpler system would serve as a better basis for the authors' statement "that sedimentation analysis is potentially far more powerful than chemical precipitation as a means of following the extent of enzymatic degradation."

Currie & Bull (97) have studied the molecular weight distribution of products formed in the peptic hydrolysis of ovalbumin. The spread monolayer technique was applied in the analysis. The authors reach the conclusion that the peptides formed roughly fall in two classes, one with molecular weights less than 2000 and one with molecular weights higher than 30,000. Intermediate weight classes are present in smaller amounts. Values of pH in excess of 2, low temperature, and high pepsin concentration favor the accumulation of low molecular peptides. The general results indicate that ovalbumin, before it can be digested by pepsin, must undergo an activation which may be regarded as a form of denaturation.

Anfinsen (98) has investigated the peptic hydrolysis of ribonuclease. Approximately 10 bonds are split per molecule, but the resulting peptides, containing on the average seven to eight amino acids per mole, do not readily disperse but seem in part to retain the secondary structure characteristic of the ribonuclease molecule.

Korsgaard Christensen (99) has made an elaborate study of the enzymatic

breakdown of β-lactoglobulin and ovalbumin by trypsin, α-chymotrypsin, and pepsin. His interest was centered on the initial stages of proteolysis and the part played by denaturation in the opening of the molecule to the endopeptidase attack. Enzyme action was studied in the presence of urea or at extreme pH-values, whereby denaturation, measured by the change in optical rotation, is accelerated in proportion to peptide bond hydrolysis. Korsgaard Christensen's experiments show that the reactions between different enzymes and substrate proteins may be highly different as regards paths of breakdown, but that denaturation in a number of cases precedes endopeptidase action also in the absence of denaturing agents where it often is the bottle neck in the proteolytic process. As for details we refer to the original.

On the basis of experiments in which the action of α-chymotrypsin upon native and denatured proteins was compared with that of chymotrypsin B (100), Ambrose & Laskowski (101) suggest that the denaturation which may precede the endopeptidase activity in the process of digesting native lysozyme is facilitated more by the former than by the latter enzyme.

Lennox (102) has studied the dissolution of purified native wool by different enzymes. Sodium bisulfite and a series of denaturing agents such as urea, thiourea, guanidine, acetamide etc., were added in varying amounts. Papain, which is most resistant to reducing conditions and to denaturation by urea, appeared to be the most powerful agent.

Orekhovich, Tustanovskii & Orekhovich (103, 104) have studied the enzymatic hydrolysis of crystalline skin proteins. Banga (105) and Banga & Nowotny (106) suggest that the solubilization of elastin by elastase is a depolymerization without cleavage of peptide bonds. A proof of this hypothesis is, however, very difficult and has not been given. We may refer to a paper by Schauenstein & Stanke (107) dealing with peptic hydrolysis of collagen.

According to Gorini & Audrain (108) the action of trypsin upon serum albumin may be explained on the basis of the assumption that the latter is an equilibrium mixture of two forms, one of which is resistant to trypsin. The inhibiting action of ions like Ca^{++} and Mn^{++} is in part explained by assuming that the ions displace the equilibrium in favor of the resistant form by combining with this. Similar assumptions may be applied to the tryptic digestion of lysozyme [Gorini & Felix (109)].

Abrams & Jacobsen (110) have observed that a proteinase from *Bacillus subtilis* is able to activate chymotrypsinogen. At 0°C. and low pH, activation proceeds with little accompanying proteolysis (as in the tryptic activation). At higher temperature and high pH, the proteolytic breakdown is accelerated and substantial loss of activity occurs. According to Szent-Györgyi (111) trypsin digests myosin without affecting its adenosinetriphosphatase activity. There is an initial reaction involved in which the myosin molecule is split in two. One moiety (40 to 45 per cent) can be crystallized and is devoid of adenosinetriphosphatase activity, the other combines with actin and has the whole adenosinetriphosphatase activity of the original myosin.

A series of interesting papers on the thrombin-catalyzed transformation of fibrinogen to fibrin have appeared during 1951–52. As regards the physical changes involved we may quote papers by Laki (112), Edsall & Lever (113), Waugh & Livingstone (114), and Ehrlich, Schulman & Ferry (115). It appears that the physical change accompanying the transformation is small if thrombin is allowed to act on fibrinogen under conditions where clotting does not occur (low pH, concentrated urea solutions). The difference between the size and shape of the molecules of fibrinogen on one hand and of fibrin on the other is therefore small when the marked tendency of the latter to polymerize is counteracted by the conditions of the medium. Thrombin, like rennin in milk coagulation, is not involved in the clotting process proper but is active in a chemical reaction preceding the clotting (if the conditions for clotting are present). According to Lorand *et al.* (116 to 119) this chemical process is a proteolytic hydrolysis whereby a peptide is liberated [see also Laki (120)].

The fibrinogen molecule has two N-terminal tyrosines and one glutamic acid, while that of fibrin has four N-terminal glycines but no glutamic acid. The peptide (or peptides) liberated constitutes about three per cent of the fibrinogen and is assumed to contain the terminal glutamic acid lost in the transformation. Peptide bonds in which the amino group of glycine is involved are apparently split by thrombin.

The question of the homogeneity of thrombin has been raised again by Kowarzyk (121) who maintains that the clotting and the liberation of non-protein nitrogen are effected by two different enzymes, the thrombin proper and a thrombin protease which latter is activated under "strictly the same conditions as are essential for the conversion of prothrombin to thrombin." A note added in proof indicates that a partial separation has been accomplished.

Although the relationship between enzyme and substrate is a more distant one in the ovalbumin-plakalbumin transformation, we may briefly mention some recent developments in the investigation of this system which is reminiscent of the fibrinogen-fibrin as well as of several precursor-enzyme transformations. By means of the Edman method (122, 123), Ottesen & Wollenberger (124) have made it likely that the three peptides liberated during the action of the *Bacillus subtilis* proteinase [compare Ottesen & Villee (125)] have the compositions Ala-gly-val-aspa-ala-ala, Ala-gly-val-aspa, and Ala-ala. Since the two latter peptides seem to be parts of the former in the ovalbumin molecule, the enzyme is therefore able to split a bond between aspartic acid and alanine. As regards possible mechanisms of the over-all reaction, reference is made to (126). We should like to add that the presence of *Bacillus subtilis* (or related organisms) in all unsterilized biological systems make the enzyme studied here a possible contaminant of considerable importance in investigations of enzymatic specificity.

Rovery & Desnuelle (127, 128) have investigated the liberation of free amino acids in the hydrolysis of horse globin by crystalline enzymes. They report that, per mole of substrate, 1 mole of phenylalanine, 0.25 mole of

lysine, and 0.1 mole of arginine were liberated by pepsin, trypsin, and chymotrypsin respectively. Other free amino acids could not be detected.

As an example of the enzymatic hydrolysis of a synthetic protein-like substrate we may mention the tryptic breakdown of poly-L-lysine studied by Waley & Watson (129). In conformity with investigations by Katchalski (130), and from what is known about the specificity of trypsin, these authors find that an adjacent carboxyl or amino group prevents the action of this enzyme. Hence free L-lysine is not formed. Green & Stahmann (131) have studied the enzymatic hydrolysis of glutamic acid polypeptides. Pepsin, trypsin, and chymotrypsin had no effect while carboxypeptidase and papain were active.

General Enzyme Theory

The recent development of the theory of enzyme action has been very interesting to follow. The majority of workers have centered their interest on what is called the "active group" of the enzyme, its structure in relation to the substrate attacked. They assume the presence of enzyme-substrate complexes (ES) formed through intimate contact between the two molecules, a "lock-and-key-system" based on short range forces, or actual chemical bonds between the active group and the substrate. The rate of the process, $ES \rightarrow E + R$, is assumed to be much higher than that of $S \rightarrow R$, by mechanisms that are as yet insufficiently known. Swain & Brown (132) have given an interesting discussion of the concept of polyfunctional catalysis and have carried it into enzyme chemistry using the model illustrated in Figure 1. Two groups of the enzyme, one nucleophilic, the other electrophilic, are spatially so arranged that a simultaneous action upon the ester RCOOR′ and the amine R,R,′NH can take place causing an exchange of an alcohol group with an amine.

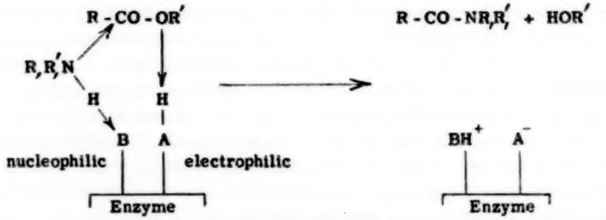


FIG. 1. Model of Enzyme Action

If R′ and R,′ are replaced by H the reversed reaction is hydrolysis of a peptide bond. The authors mention free amino groups and carboxyl groups of the protein as possible examples of B and AH respectively, but do not emphasize the unfavorable conditions for simultaneous existence of these groups in aqueous solution.

An interesting model of a somewhat different kind is found in the reaction of Levy (133) (Figure 2). All reactions in this scheme are known to occur in

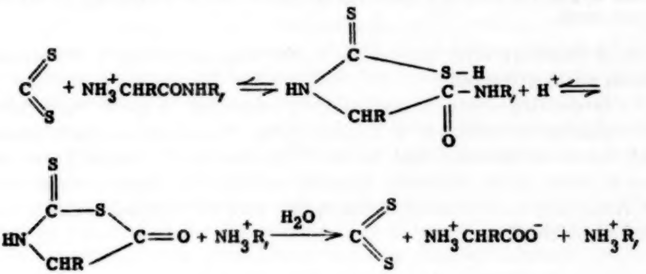
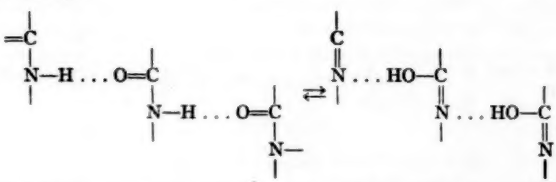


FIG. 2. Model of Enzyme Action

aqueous solution at pH 7 to 8, but some of the steps are very slow and side reactions complicate this otherwise simple system imitating the action of an aminopeptidase.

In most of the discussions of the nature of the active group of proteolytic enzymes the role played by the vast structure of the protein molecule "carrying" the active group is discussed in rather vague terms, and our knowledge is admittedly unsatisfactory on this point. Mention should, however, be made of an article by Leach & Lindley (134) which is based on the electron flow hypothesis suggested by Szent-Györgyi (135) and Wirtz (136), and discussed by Evans & Gergely (137) and Geissmann (138). This hypothesis ascribes the catalytic power of enzyme proteins to a resonance system of the following type



in which a "flow" of π-electrons may be directed to the active center of the protein molecule.

According to Leach & Lindley the enzymic proteins differ from the nonenzymic ones with regard to amino acid composition. Especially the ratio

$$(\text{aspa} + \text{aspNH}_2 + \text{gluta} + \text{glutNH}_2)/(\text{ser} + \text{threo})$$

is much lower (ca. 1) in enzymic than in nonenzymic proteins (between 2 and 3). This difference in amino acid pattern seems to be significant and therefore represents a new observation of considerable interest. We are, however, unable to judge whether on the present basis the authors are justified in assuming

the existence in enzymic molecules of frequent interchain hydrogen bonds probably largely of the carboxyl-hydroxyl type, which reinforce the intrachain hydrogen bonding of peptide links to form a highly resonating network over which electron flow can occur.

We shall therefore refer the reader to the original, where a few details of electron paths are given.

An interesting study of the catalytic properties of α-chymotrypsin has been published by Doherty & Vaslow (139). This enzyme, the ubiquity of which has been demonstrated by the Neurath school, has only one active group in its molecule, probably, but not certainly, involving a serine residue (44). According to Neurath & Schwert (92, 140, 141) its substrates are characterized by the formula

$$R{-}CO{-}NH{-}C(R')H{-}CO \,\vdots\, {-}R''$$

where R may be CH_3 or C_6H_5; R′, $CH_2C_6H_4OH$ or $CH_2C_6H_2Br_2OH$ or $CH_2C_6H_5$; while R″ may be OR_1 or NHR_2. Hydrolysis of the bond between C and R″ requires that the configuration around the central carbon atom corresponds to the L-form of the amino acid involved. Now according to Sprinson & Rittenberg (142) and Doherty & Vaslow, R_1 may be represented by hydrogen so that reactions of the type—$COOH+H_2O^{18}=$—$COO^{18}H + H_2O$ are catalyzed by the enzyme. Substances like Cbz-L-phenylalanine (Sprinson & Rittenberg) or acetyl-3,5-dibromo-L-tyrosine (Doherty & Vaslow) therefore exchange their carboxyl oxygens according to the above scheme and may be considered as substrates for α-chymotrypsin.

The process is visualized conjecturally in Figure 3.

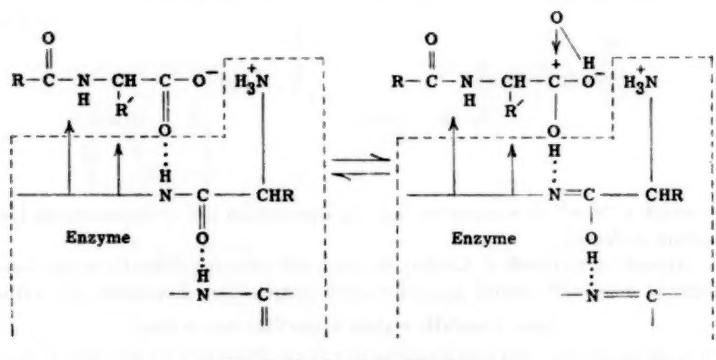


FIG. 3. Mechanism of Exchange.

This catalytic system is very well suited for investigations of the thermodynamic functions of the binding process since it is almost "static." By

means of the dialysis equilibrium technique of Klotz, Doherty & Vaslow have studied the binding between chymotrypsin and the above mentioned substrate at different pH and temperature. Substrate concentration was determined by labeling with Br^{82} and measuring radioactivity.

Apparent values of the functions ΔF, ΔH, and ΔS were determined, and a high negative value for ΔS was found in the pH region where catalytic activity is expected. The same decrease in entropy is not observed if chymotrypsin is replaced by chymotrypsinogen or if substrates with D-configuration are used (142a). It is therefore likely that the binding of substrate to enzyme causes the number of vibrational states of the latter to decrease.

In concluding this section we may touch upon the experiments by Rothen (143) who has given a further contribution this year to the field developed by him: that of long distance forces and long distance catalytic induction. Due to the complexity of the problem this work has been without adequate theoretical support, and it therefore rests almost entirely on the evidence of a very intricate experimental technique. To the primitive mind (which we regret to represent) there is no reason, a priori, for rejecting the idea that enzyme molecules emit radiation which may excite substrate molecules at some distance and cause reactions to occur. Naturally any system at ordinary temperature is filled with infrared radiation emitted by its molecules, and the question is only whether high frequency radiation from the enzyme proteins ($\nu = 10^{14}$ sec.$^{-1}$ corresponding to 10 kcal.) is sufficiently dense to give a measurable probability of excitation at distances of several diameters of a water molecule. In homogeneous enzymatic catalysis involving low-molecular substrates the number of free substrate molecules which react in this way will probably be completely negligible in comparison to the number reacting in close oriented contact with the enzyme protein. However, in heterogeneous catalysis where both substrate and enzyme are represented by high-molecular oriented layers the situation may be different. We refer the reader to recent publications pertaining to the theoretical side of the question of long distance forces [Jehle (144); Kirkwood & Shumaker (145)].

Synthesis

Thermodynamics.—Until recently our knowledge about the thermodynamics of peptide synthesis was based upon the careful work of Huffman. The number of amino acids involved in the compounds studied by him was however small, and it is therefore of importance to extend such investigations to include longer peptides containing a greater variety of amino acids. Breitenbach, Derkosch & Wessely (146, 147) have made some studies of the heat of formation of polyphenylalanine and polysarcosine as prepared by the carbamic anhydride method. They find the value $\Delta H/n = -7.1 + 68.3/n$ kcal. for the reaction

$$n \times \text{phenylalanine (solid)} \rightarrow \text{polyphenylalanine (solid)} + (n-1)H_2O \text{ (liquid)}$$

where n is the average number of residues per polypeptide chain. Since n is

supposed to be large (no figure is actually given) the process is exothermic in contradistinction to that of glycylglycine formation [2 glycine (solid)→glycylglycine (solid)+H_2O (liquid)] where $\Delta H/2 = -31.05+68.3/2 = +3.1$ kcal. The result is interesting, but since the corresponding entropy term is undetermined and the solubility of the polymer in aqueous solutions is unknown, it is too early to draw conclusions regarding its significance for the problem of protein synthesis by proteolytic enzymes. A low solubility of the synthetic product will explain a negative ΔF for the solid-solid system without additional assumptions regarding the energetics of the peptide bond formation between phenylalanines in aqueous solution.

The approach by Fruton and his school (148, 149) to the same problem is highly interesting. By combining equilibrium determinations by means of their tracer technique with calorimetric measurements of the heat of reactions [Dobry & Sturtevant (150)] Dobry, Fruton & Sturtevant (151) have determined the thermodynamic functions of the process

benzoyl-L-tyrosylglycine amide (1 M) + H_2O
→ benzoyl-L-tyrosine (1 M) + glycine amide (1 M)

which is catalyzed by chymotrypsin in aqueous medium.

They found $\Delta F = -0.42$ kcal., $\Delta H = -1.55$ kcal. and $\Delta S = -3.8$ kcal./degree per mole. The value of ΔF is about a power of 10 smaller than that found for dipeptides with charged end-groups and shows that synthesis is greatly facilitated if these charges are removed by substitution, as in longer peptides or in acylated and esterified or amidated peptides.

Plain synthesis catalyzed by proteolytic enzymes.—Synthesis of sparingly soluble compounds has been studied by several authors. Schuller & Niemann (152) report synthesis of the phenylhydrazides of benzoyl-L-tryptophan, benzoyl-L-tyrosine and benzoyl-L-phenylalanine, the catalyst being α-chymotrypsin. The influence of pH and of buffer ions was studied [compare also (153)].

Fox & Winitz (154) have studied the yields in the papain- and ficin-catalyzed synthesis of benzoyl amino acid anilides after 72 hours at 40°C. No attempts were made to determine equilibrium yields, and the solubility of the anilides formed was not investigated. The conclusions drawn by the authors concerning detailed enzyme specificity can therefore only be roughly qualitative. Enzyme-catalyzed synthetic reactions of this type are however useful in attempts to separate mixtures of different substances. We may refer to a recent paper by Yoneya (155) in which the optical isomers of methionine and lysine were separated in this way [enzyme ficin; see also (156, 157)].

Laskowski, Rakowitz & Scheraga (158) report experiments which they regard as indicating that a thrombin-catalyzed synthesis of fibrinogen from fibrinopeptide (118, 119, 120) and fibrin clots may take place. Since the situation is almost the opposite of that considered above, a soluble synthetic product being formed from a mixture of a soluble and an insoluble substance, the problem is theoretically interesting. However, as pointed out by the

authors the evidence is very weak and more experiments are needed.

Finally, mention should be made of the synthesis under high pressure reported by Bresler and his group (159, 160). The systems worked with were aqueous solutions of serum albumin, ovalbumin, or insulin deeply digested (up to 19 per cent hydrolysis of peptide bonds) with crystalline trypsin or mixtures of this enzyme with crystalline chymotrypsin. Synthesis in these digests was effected at 6000 atm.,—the same enzymes, stabilized by 20 per cent glucose, acting as catalysts. Synthesis was followed by centrifugal analysis, and in one case (serum albumin) crystallization of what is assumed to be native resynthesized protein is reported. The sedimentation diagrams published (159) show unquestionably the formation of material of the same molecular weight class as the native protein, but even in the case where 98 per cent resynthesis is postulated the material is evidently inhomogeneous. The authors admittedly do not assume complete identity with the original protein of that crystallized after pressure treatment, and this has sharpened our interest in the work. In its continuation we propose more controls for undigested protein, for glucose bound to protein etc. Furthermore, experiments on simpler systems are called for.

Transpeptidation.—A series of papers have appeared this year on reactions of the type

$$RCO—NHR_1 + NH_3^+R_2 \rightleftarrows RCO—NHR_2 + NH_3^+R_1$$

or

$$RCO—OR_1 + NH_3^+R_2 \rightleftarrows RCO—NHR_2 + R_1OH$$

in which ΔF is probably not far from zero. Especially through the work of Fruton *et al.* [see (149)] it has been shown that proteolytic enzymes can act as catalysts in such reactions. A great number of different reactions catalyzed by chymotrypsin, papain, beef cathepsin C (48), and ficin have been investigated. As an example we may mention the reaction

glycyl-L-phenylalanine amide + L-arginine amide
→ glycyl-L-phenylalanyl-L-arginine amide + NH_3

catalyzed by beef cathepsin C at pH 7 (161). After 1 hr. at 38°C. and at the given conditions, 24.4 per cent of the components had combined while 19.8 per cent had been hydrolyzed, which is admittedly a high yield of transpeptidation. Mention should also be made of the cathepsin-catalyzed polymerization reaction of glycyl-L-phenylalanine amide in which a chain of approximately eight glycyl-L-phenylalanyl residues is formed with liberation of ammonia. The synthetic polymer is insoluble. The reaction occurs only at pH 7 and not at pH 5 where hydrolysis predominates. In recent work (149) a ficin-catalyzed polymerization is reported leading to carbobenzoxy-L-glutamyl-(L-methionyl)$_{10}$-L-methionine amide. Furthermore, reference should be made to a paper by Dowmont & Fruton (162) where a method for identifying transpeptidation products is described, and other high yield reactions are reported.

Formation of insoluble polymers by the action of chymotrypsin upon peptic digests has been described by Tauber. This year he reports that the same enzyme catalyzes the formation of L-phenylalanyl-L-phenylalanine ethylester from the amino acid ester (163). The experiments are similar to those of Brenner *et al.* (164). In recent investigations (165, 166) the latter authors report formation of D-methionyl-D-methionine isopropylester from the amino acid ester. Since the catalysts used were liver homogenates, autolyzates, or mitochondria it is doubtful whether these interesting papers should be reviewed in the chapter on proteolytic enzymes. The same is true of a number of very important contributions to the problem of general biosynthesis of proteins. We therefore limit ourselves with regret to mentioning a few articles of Waelsh (167), Hanes, Hird & Isherwood (168), Borsook (169), Bloch, Snoke & Yanari (170), Binkley & Christensen (171). Especially those of Waelsh (on glutamo- and aspartotransferases) and of Hanes *et al.* (γ-glutamyl transpeptidase from kidney and glycyl transpeptidase from cabbage leaves) have a special bearing on the problem discussed in this section.

Analytical Chemistry

Methods for enzyme determination.—No essentially new methods for protease determination have been published during the past year. It is clear that with the development of new methods for identification of protein splitproducts these methods would be applied analytically [analysis of protein digests by the Stein & Moore starch column technique: Lens & Evertzen (172); serum peptidase determination by filter paper chromatography: Hanson & Wenzel (173)]. In routine proteinase determination the methods involving separation of "digested" and "undigested" protein are completely dominant, unquestionably because of the presence in almost every laboratory of standard spectrophotometers by which the "digested" protein may be easily determined. There is no doubt that some of these methods (e.g., that of Anson) both with regard to speed and accuracy are superior to methods where the chemical change, the splitting of peptide bonds, is directly measured, but it should be mentioned that these advantages are gained at the expense of the simplicity of the analytical results. For this reason critical investigations like that of Wallenfels (74) may be of value.

Constitution determined by means of enzymes.—This field has developed greatly in later years. Since the path of breakdown, e.g. of proteins, may also serve to characterize the enzymes employed as agents, several of the problems treated here may have been dealt with in the section on Chemistry of Action (p. 63). In the papers now to be discussed the specificity of the enzymes employed in the majority of cases is taken as known from previous work; we shall, therefore, give a brief review here of some of the more important results and leave the detailed treatment to the authors of the chapter on proteins.

Sanger & Thompson have determined the amino acid sequence in the

glycyl chain of insulin (174) [see also Sanger & Tuppy (175); Sanger, Thompson & Tuppy (176)]. The enzymes used in this important work were chymotrypsin, carboxypeptidase (treated with DFP[2] to inactivate traces of chymotrypsin), pepsin, and papain. New features of enzymatic interest are that the bond $CySO_3H$—Ser (bond 11–12 in the glycyl chain) is split by chymotrypsin; that bonds between two aromatic amino acids are readily split by pepsin [compare Baker (64)]; and that the bond between glutamic acid and asparagine is split by pepsin.

Results of Harris (177) on the effect of carboxypeptidase upon insulin are in agreement with the findings of Sanger, Thompson & Tuppy. Harris & Li (178) have confirmed the results of Sanger & Tuppy on the tryptic breakdown of insulin. Only two bonds in the phenylalanyl chain are split by this enzyme, and in both the carboxylic group of basic amino acids, viz. arginine and lysine, is involved.

The action of carboxypeptidase has been studied by several authors. According to Thompson (179), leucine is the C-terminal residue of lysozyme as determined by this enzyme. Condliffe & Li (180) have identified serine, alanine, and phenylalanine in significant amounts among the split-products formed by the action of carboxypeptidase upon crystalline growth hormone. Harris & Knight (181) have observed abundant release of threonine from tobacco mosaic virus [3400 residues per mole ($M = 5 \times 10^7$)] without loss in infectivity [for other applications see Waldschmidt-Leitz & Gauss (182)].

Miller & Neuhaus (183) have studied the peptic breakdown of acetylated ovalbumin. Tsou (184) has studied the digestion of cytochrome-*c* by various proteolytic enzymes. Since these interesting experiments do not throw light upon the action of the enzymes employed, we refer the readers to the original.

Preparation of specific substances by enzymes.—In this brief section we may refer to papers by Mendenhall & Li (185), Li, Ash & Papkoff (186), and White, Fierce & Lesh (187) (adrenocorticotropic peptides), Sloane & McKee (188) (growth stimulatory factor for *Staphylococcus albus*), and similarly, for simpler substances, to papers by Greenstein *et al.* (189, 190, 191), Berlinguet & Gaudry (192) and Nishio (193), all on resolution of mixtures of optical isomers by enzymes (acylases).

BIOLOGY

Histo- and cytochemistry.—Pope (194) has made an extensive investigation of the quantitative distribution of dipeptidase in architectonic layers of rat cerebral cortex. The activity of dipeptidase (substrate DL-alanylglycine) runs roughly parallel with the mass of the tissue, which suggests that the nerve cell bodies are the principal sites of this enzyme. Cholinesterase with which the peptidase is compared is primarily localized at the surfaces of dendrites and axones [compare Ansell & Richter (195)].

Holter & Pollock (196) have studied the distribution of dipeptidase in the plasmodium of *Physarum polycephalum.* Since centrifugation had little or no effect on the distribution, the authors conclude that dipeptidase is not

bound to any granular component [compare Holter & Løvtrup (197): distribution of peptidase and proteinase in *Chaos chaos*].

Kaufmann (198) has studied the effect of trypsin on salivary gland chromosomes. According to the author no dissolution of the chromosomal proteins occurs although swelling, ascribed to a hydrolysis of the protein, is observed. What happens is probably that peptide bonds in which arginine or lysine carboxyls are involved are hydrolyzed, but that after hydrolysis the basic groups of these amino acids, with the protein fragments they are part of, recombine with the nucleic acid phosphate groups in a loose fashion permitting water to intrude. It depends then upon the size and physical state of these fragments whether or not they can be "dissolved" by exchange reactions with cations from the medium [compare Anfinsen (98)].

Embryology.—Gustafson & Hasselberg (199) have studied the proteinases of developing sea-urchin eggs. With denatured hemoglobin as a substrate, splitting was observed at all developmental stages. With benzoylarginine amide no splitting was found up to the mesenchyme blastula stage after which a pronounced rise was observed (cysteine was added as an activator). Emanuelsson (200) has studied the proteolytic activity of hens' eggs prior to incubation. The yolk was rich in proteinase.

Microbiology.—Maxwell (201, 202) has made an extensive study of the growth conditions required for *Aspergillus oryzae* to produce proteolytic enzyme. Gillespie & Woods (203, 204) report the isolation of two proteinases from cultures of *A. oryzae*. They find their isoelectric points at pH 4.1 and 4.8. In a series of papers by Dion (205, 206), by McConnell (207), and by Muggleton & Webb (208) elaborate studies have been made of the liberation and properties of exocellular proteinases from different species of fungi and actinomycetes. Some of these enzymes seem to resemble the above mentioned enzymes [compare Wallenfels (74), and Pechmann (73)] while others may be more closely related to the proteinase described by Güntelberg & Ottesen (59).

The collagenases of bacteria have been studied by Brisou [(209, 210), different bacteria] and Narayanan & Menon [(211), *Vibrio cholerae*]. Brisou points to a similarity between this enzyme in *Cl. histolyticum* and papain. We may refer to Ogle's paper (63), quoted elsewhere.

Comparative biochemistry.—In a series of papers, Day (212) and Waterhouse (213) have studied the digestion of wool by insects, especially by the larvae of *Tineola bisselliella* (clothes moth). In general they have confirmed and greatly extended the old work by Duspiva & Linderstrøm-Lang (214, 215) who suggested that the keratin of the wool was reduced in the intestine of the larvae and split by an alkaline proteinase which was little sensitive to the SH groups formed in the reduction process. The mechanism by which reduction occurs is still obscure.

By means of a microtechnique based on digestion of sulfanilimide-azocasein and determination of nonprotein color, Fisk & Shambaugh (216) have studied the protease activity of the midgut of *Aedes aegypti* mosquitoes

in relation to feeding. After a fall in activity which follows immediately after feeding the starved mosquitoes with blood, a rapid increase is observed during the ensuing hours, until a 26-fold increase (above the residual value of unfed mosquitoes) is attained [see further Fisk (217)].

Cavier (218) has made a preliminary investigation of the proteinase of *Ascaris lumbricoides*. The pH optimum was found to be around 6. Carpenter (219) has studied the peptidases of the intestinal mucosa of the same nematode. The substrates used were di- and tripeptides of glycine, alanine, and leucine. Besides dipeptidase two aminopeptidases are present, one activated by Mn^{++}, the other not. These enzymes are localized mainly in the anterior part of the gut.

Utzino, Onoyama & Nakao (220) have studied the influence of benzene hexachloride on toad liver cathepsin. The proteinase from bullfrog liver was studied by Ono (221) and various tissue proteinases from the Japanese fish *Ophiocephalus tadiana* by Utzino & Nishiwaki (222). Takaoka *et al.* (223, 224) have made a broad investigation of the gastric enzymes of various marine mammals, whale, dolphin, seal, and furseal. They demonstrate the presence of two enzymes with pH-optima 1.4 to 1.6 and 3.4 to 3.6. The latter enzyme, which they name β-pepsin, seems to be reminiscent of the gastric cathepsin of Merten *et al.* (47).

Adams, McFadden & Smith (225) have compared erythrocyte peptidases in man and other species.

Danielsson (226) has made an extensive study of the proteins in seeds of Gramineae and Leguminoseae. The proteinase activity follows the albumin fraction, and the enzyme could be extracted with water and purified by salts.

Jansen, Jang & Balls (227) have studied the aminopeptidases of the peel of citrus fruit. The peptides alanylglycine, alanylalanine, glycyltryptophan, glycylleucine, leucyldiglycine, and alanyldiglycine were rapidly split; dehydropeptides and D-amino acid peptides were also hydrolyzable. The pH-optima varied from 7 to 7.3. No metal activation was observed and no inhibition by DFP.[2]

The enzyme ficin and other plant proteases have been the subjects of numerous papers by Japanese workers. We may refer to (157, 228, 229, 230).

General Reviews

The reader will find in references 231 to 240 a number of recently published reviews on proteolytic enzymes.

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NONOXIDATIVE AND NONPROTEOLYTIC ENZYMES: GLYCOSIDASES[1]

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Widely different enzyme types must sometimes be forced into an uneasy proximity within the negative category "Nonoxidative, Nonproteolytic Enzymes" traditional to this Annual Review. A wide range of nonproteolytics and nonoxidatives was covered in the preceding year. The present review, relatively restricted in its scope, concerns only one large group, that of the glycosidases.

SYNTHESIS AND CLEAVAGE OF AMYLOSACCHARIDES

The amylosaccharides include both linear polymers (amyloses) whose representative of lowest molecular weight is maltose, and branched polymers (glycogens, amylopectins, dextrans) whose representative of lowest molecular weight is the trisaccharide panose (1, 2).

Maltose

The view that the interglycosidic linkage in maltose, like the same linkage in higher members of the amylose class, can be degraded enzymatically by means other than hydrolysis (3) has been confirmed repeatedly.

Maltose phosphorylase.—A postulated phosphorolytic degradation pathway for maltose has been demonstrated in *Neisseria meningitidis* by Fitting & Scherp (4). Although *Neisseria* cell extract failed to effect uptake of inorganic phosphate in the presence of glucose, it did so in the presence of maltose. The products of maltose breakdown were a reducing sugar and an alkali-stable acid-labile hexose phosphate ester. Fitting & Doudoroff (5) have identified the ester as β-glucose-1-phosphate. Synthetic β-glucose-1-phosphate and glucose were condensed by the cell extract and afforded a disaccharide with the properties of maltose, whereas α-glucose-1-phosphate and glucose were not condensed. The reversible reaction mediated by maltose-phosphorylase may thus be formulated as follows:

$$\text{maltose}+\text{inorganic phosphate}\rightleftarrows\beta\text{-glucose-1-phosphate}+\text{glucose.}$$

The remarkable feature of the reaction is the inversion of the configuration of the glucosidic linkage. This illustrates the difficulties of inference concerning the configuration of a saccharide from the configuration of a product obtained by enzymatic means. It is of interest to recall that a similar case of

[1] The survey of the literature pertaining to this review was concluded in September, 1952.

configurational inversion is observed in the hydrolytic degradation of amylose chains by β-amylases.

Polymerative maltolysis.—A polymerative degradation of maltose is mediated by the enzyme amylomaltase whose existence in a mutant of *Escherichia coli* was first reported by Monod & Torriani (6) and shortly thereafter by Doudoroff *et al.* (7). In a careful study, Barker & Bourne (8) have separated some of the oligosaccharides formed from maltose by Monod's strain of *E. coli* and have characterized these products by paper chromatographic analysis, methylation techniques of end-group assay, and by oxidation with hypoiodite and periodate. The work of Barker & Bourne has shown that the products of the amylomaltase action form an unbroken amylose homolog series at least up to the polymerization degree $n=5$. The view that amylomaltase acts by repetitive stepwise transfer of single glucosyl units from the maltose donor to the amylose acceptors is substantiated by this finding. The presence of amylotriose in the oligosaccharide fraction supports the contention that maltose as such can act as a glucosyl acceptor.

Still another transfer enzyme converts maltose into a group of oligosaccharides with interglucosidic linkages at C-6 positions. The existence of an enzyme of this type in *Aspergillus niger* was suggested by Pan, Andreasen & Kolachov (9) on the basis of their finding that filtrates of a strain of *A. niger* produce nonfermentable carbohydrates from maltose. The work of Wolfrom *et al.* (1) and French (2) has identified the trisaccharide panose, namely 4-α-isomaltosyl-D-glucose, among the products of the reaction. An elegant study by Pazur & French (10, 11) has established the presence of four C-6 substituted saccharides in the nonfermentable carbohydrate fraction. The enzyme system from *A. oryzae* was partly freed from amylases and maltose hydrolase by treatment with starch. Isomaltose, panose, dextrantriose, and 4-α-dextrantriosyl-D-glucose were identified among the reaction products. With C^{14} glucose as the acceptor and unlabelled maltose as the donor, radioactivity was recovered in isomaltose and dextrantriose but not in maltose and panose. The following reaction scheme is a paraphrase of that advanced by Pazur & French:

$$\begin{array}{lll}
G'G & +\text{enzyme} \rightarrow G\text{:enzyme}+G & \\
G\text{:enzyme}+G^{*} & \rightarrow G''G & +\text{enzyme} \\
G\text{:enzyme}+G'G & \rightarrow G''G'G & +\text{enzyme} \\
G\text{:enzyme}+G''G^{*} & \rightarrow G''G''G^{*} & +\text{enzyme} \\
G\text{:enzyme}+G''G'G & \rightarrow G''G''G'G & +\text{enzyme}
\end{array}$$

Where G is glucose, G^{*} is labelled glucose, G' is α-glucosyl substituted in C-4 of G, G'' is α-glucosyl substituted in C-6 of G, and G:enzyme represents a hypothetical glucosyl-enzyme derivative. The name isomaltomaltase might be appropriate for this new enzyme.

For the first time, a system for glucosyl transfer from maltose has also been observed in an animal tissue. Whitby (12) showed that a rat liver enzyme forms riboflavinyl glucoside from riboflavin and maltose although it fails to condense riboflavin and glucose. The reaction is thought to consist of

the transfer of a glucosyl group from donor maltose to the terminal hydroxyl group of the ribityl residue of acceptor riboflavin.

Maltose hydrolases.—In regard to the hydrolytic mechanism of maltose breakdown, the view was long entertained that maltose hydrolysis may be effected in yeast by a single enzyme α-glucosidase, which was supposed to be capable of hydrolyzing alkyl and aryl α-glucosides as well as different oligosaccharides having a terminal α-glucosidic moiety (cf. 13). Re-examination of this view has now become necessary.

Genetic analyses on yeast strains which fermented α-methylglucoside or maltose but not both these substrates were conducted by Lindegren & Lindegren (14) and have indicated that distinct separable genes respectively control maltose and α-methylglucoside fermentation. Hestrin & Lindegren (15) recovered from yeast of genotypes which fermented α-methylglucoside specifically an enzyme which hydrolyzed α-methylglucoside but evinced little or no hydrolytic activity towards maltose and melezitose. Furthermore, Spiegelman, Sussman & Taylor (16) have devised a fractionation procedure whereby yeast maltase and an α-heteroglucosidase (α-methylglucosidase) occurring together in the original cell extract could be separated.

In the hybrid strains studied by Hestrin & Lindegren (17), the induction of the production of either the α-heteroglucosidase or the maltase by their specific substrates was found to be attended by the induction of a hydrolytic activity towards sucrose without concomitant appearance of a hydrolytic activity towards raffinose. It seemed reasonable to suggest that the maltase and the heteroglucosidase are capable as such of sucrose hydrolysis or can be transformed by the cell into sucrase.

The problem of maltose metabolism is clearly much more complex than has at times been supposed. The discovery by Winge & Roberts (18) that there are at least four distinct genes any one of which separately suffices to confer ability to ferment maltose poses the question whether these genes replace each other in the same process of enzyme synthesis, mediate distinct reactions which lead independently to the same enzyme product, or mediate reactions which bring about syntheses of different maltases.

The work of Halvorson & Spiegelman (34) has thrown light on the type of the reactions which may be involved in the adaptive response to maltose. In a survey of the effect of halogenated phenylalanine derivatives on the growth of *Saccharomyces cerevisiae*, they have found a correlation between the ability to inhibit growth and the inhibitory action on the response to maltose. The inhibitors hampered incorporation of different amino acids from the free amino acid pool into the cell protein. Taking the view that the utilization of the free amino acids is directly concerned in the formation of maltase, Halvorson & Spiegelman suggest that the primary step of the adaptive response to maltose involves several amino acids.

Amylose, Amylopectin, Glycogen, and Dextran

Z-enzyme.—A startling outcome has resulted from the study of the limit of the action of sweet potato crystalline β-amylase on amylose by Peat, Pirt,

& Whelan (19, 20). The limit dextrin resulting from the action of the sweet potato β-amylase on potato amylose was found to be a high molecular polysaccharide giving a blue stain with iodine. Whereas the sweet potato β-amylase had failed to degrade potato amylose beyond a 70 per cent arrest point, essentially complete conversion of this amylose was effected by an amorphous soya bean β-amylase preparation. Since these enzyme sources do not show a corresponding disparity when amylopectin is the substrate, their difference cannot be ascribed to contamination of the soya enzyme by an α-amylase. Exposure of crude soya bean preparation to pH 3.0 at 35°C. led to the recovery of a factor (Z-enzyme) whose activity allowed the complete degradation of amylose by enzyme systems (phosphorylases and β-amylases) which had failed to degrade amylose beyond a 70 per cent arrest point in absence of Z-enzyme (21). The Z-enzyme was phosphatase-free. Its action on amylose led to a slight loss in viscosity and to liberation of a small amount of glucose. This was in accord with the view that the action of Z-enzyme on amylose consists in a removal of glucosyl residues. It is suggested that the latter occur along the amylose chain as side branches and constitute anomalies which must be removed before the β-amylolysis or phosphorolysis of the amylose chain can be completed. Emulsins with β-glucosidase activity reproduced the effect of Z-enzyme. It has been postulated accordingly that the side branch residues of amylose possess a β-configuration.

β-Amylase.—A compromise on the controversial question whether β-amylase acts repeatedly on one amylose chain before passing to another has been urged by French *et al.* (22). These authors find that amylopentaose may be detected as a transient product of the action of β-amylase on amyloheptaose when the enzyme is allowed to act on this substrate at nonoptimal pH and temperature, whereas the amylopentaose does not occur among the transient reaction products if the reaction is carried out at an optimum pH and temperature. Similarly, salivary amylase acting on amylodextrin at unfavorable pH or temperature produced transiently amyloses with polymerization degree $n = 1$ to 8 or higher, whereas the action of the enzyme in more favorable conditions failed to form products with $n > 4$. These findings are taken as indicating that the prevalence of the single-chain mechanism reflects a situation in which an effective recombination of an enzyme particle with an amylose chain upon which it has just acted has a greater statistical probability than has the escape of the same amylose chain from the action field.

Kerr & Cleveland (115) isolated the high polymer fraction from in a reaction mixture of sweet potato crystalline β-amylase and crystalline corn amylose in which the hydrolysis had been interrupted at approximately the midpoint. On the basis of osmotic pressure measurements, ferricyanide oxidation, and intrinsic viscosity, they concluded that the isolated high polymer fraction did not differ from the original amylose in average chain length and in chain length distribution. This seemed strongly to support the view that β-amylase operated by single-chain mechanism. However, the subsequent discovery (19) that the crystalline β-amylase is incapable of de-

grading amylose beyond a 70 per cent arrest point and that this reaction produces an amylose-like high polymer as a limit-dextrin may necessitate a reconsideration of this conclusion.

The possibility that α-phenyl maltoside may serve as a substrate of β-amylase has been tested by Piquet & Fischer (25) in the context of a study of the general properties of crystallized malt β-amylase (26). Since α-phenylmaltoside proved incapable of hydrolysis by the enzyme, it may be suggested that the enzyme specificity is influenced by the structure of the chain on both sides of the terminal maltosidic linkage. This is in accord with the relatively low activity of β-amylase towards amylotriose in comparison to maltotetraose and higher amyloses and the known inability of β-amylase to remove a terminal maltosyl residue from chains in which the maltosyl group, as in the β-amylase limit dextrin of amylopectin, is attached to C-4 of a C-6 substituted glucosyl group.

According to French & Knapp (27), an enzyme from *Clostridium acetobutylicum* is an end-group type, like β-amylase and phosphorylase, and effects end-wise removal of glucosyl units from the amylose terminal by a hydrolytic process. However, the demonstrated reaction rates were of a rather low order and further work on this type of end-group amylase would be desirable. It may be noted that, in a study on *Cl. acetobutylicum* by Scott & Hedrick (28), a striking increase in amylase activity was found when amylopectin or whole starch in the growth medium was replaced by β-amylase limit dextrin.

α-Amylases.—It would be difficult in the limited confines of an annual review to do justice to the extensive and fundamental research of the late Professor Kurt Meyer and his school on the purification and properties of different α-amylases. A recent review of this work should be consulted (29). Malt amylases have been the subject of an excellent review by Schwimmer (43).

The action of salivary α-amylase on several of the lower amylose homologues has been further clarified by Roberts & Whelan (23). They have contradicted the claim (24) that maltotriose is split by salivary amylase to maltose and glucose. Whereas the end result of the action of the enzyme on maltotetraose was maltose alone, a mixture of maltose and maltotriose was found at reaction end when the action was started on amylose homologues of polymerization degree $n=5$, 6, and 7. The observed maltose/maltotriose ratio varied with the n value of the substrate in agreement with random action along the chain within the limitations set forth.

The action of α-amylase on branched substrates (amylopectin and glycogen) poses a much more difficult analytical problem. A new and unexpected element is injected by the recent announcement by Peat, Roberts & Whelan (30) that maltulose (4-[α-glucopyranosido]-fructose) is one of the products of the α-amylolysis of rabbit liver glycogen. Whelan & Roberts (62) in a chromatographic analysis of the limit dextrin produced by the exhaustive action of purified saliva α-amylase on rabbit liver glycogen found maltose, maltotriose, and a number of dextrins containing α-1:6 linkages. The

branched dextrin of lowest molecular weight was a pentasaccharide. The absence of a branched tetrasaccharide and of a C-6 substituted trisaccharide is in accord with the assumption that each of the three α-1:4-links adjacent to the branch link is resistant to hydrolysis by α-amylase.

A novel approach to the problem of the mode of synthesis of amylase has been employed by Hokin (31, 32). Out of 16 amino acids present in crystalline α-amylase, 10 were found by this investigator to be necessary and sufficient for maximum amylase synthesis in pigeon pancreas slices. Whereas amylase synthesis in the presence of the suitable amino acid mixture failed to evoke uptake of inorganic P^{32} into ribonucleic acid, specific stimulation of amylase secretion by means of carbamyl choline did lead to incorporation of P^{32} (33). These findings have suggested that ribonucleic acid is involved specifically in the secretion and transport of the enzyme rather than in its synthesis.

Phosphorylases.—Several groups of investigators have contributed to the further clarification of the nature of the phosphorylase reactions. Using defined maltohexaose as primer, Bailey & Whelan (35) have examined the effect of primer concentration, reaction time, pH, and temperature on the molecular weight and homogeneity of the product formed from α-glucose-1-phosphate by potato phosphorylase. Their study of the colored complex formed with iodine at various stages of the reaction was consistent with the conclusion that the growth of the amylose chains and their breakdown (during phosphorolysis) occur simultaneously, and that the average length of the chains varies inversely as the primer concentration. When synthetic amyloses with average chain lengths of 33, 77, and 145 glucosyl units respectively were examined with an electrokinetic ultrafiltration technique, they revealed compact distributions of the molecular weights around the above averages. The over-all equilibrium constant of phosphorolysis, the ionic species concerned, the kinetics of the process, and the effects of magnesium ion on the reaction have been the subject of careful studies by Trevelyan, Mann & Harrison (36, 37). The free energy change of the reaction, acid glucose-1-phosphate $\rightarrow$acid orthophosphate plus polysaccharide, has been calculated on the basis of the data to be -1460 cal. at 30°C.

A considerable purification of potato phosphorylase has been accomplished by Gilbert & Patrick (38) using procedures based on fractionation with alcohol, selective removal of impurities by extraction with zinc acetate solution, and further fractionation with ammonium sulfate. The resultant product has an activity nine-fold that of the best previously described preparation but is still considered to be heavily contaminated with nonphosphorylase protein, though free from Q-enzyme, amylase, and phosphatase activities.

The study of the arsenolytic decomposition of amylopectins by potato phosphorylase by Katz & Hassid (39) had suggested that this process shortens the outer chains to the same degree as β-amylolysis. Meyer *et al.* (40)

have confirmed this result. This implies that the arrest point of degradation by potato phosphorylase is different from that observed with muscle phosphorylase (41). A further test of this question using the purified phosphorylase of potato would be of interest.

It has been noted previously that the β-amylase limit-dextrins possess a very low or negligible priming activity. Meyer *et al.* (40) have found that the synthesis reaction with a β-amylase limit-dextrin as primer follows an autocatalytic course. This is thought to be a result of the circumstance that the affinity for the enzyme decreases sharply as the outer stubs of the dextrin are shortened to lengths of less than three glucosyl units. Linear amylose chains exhibit a sharp decrease in priming efficiency with decrease of chain length from four to three glucosyl units (42).

Q-enzymes.—The current view concerning the nature of Q-enzyme ascribes the formation of amylopectin from amylose to a transfer type of reaction (44, 45, 46, 47). Hobson, Whelan & Peat (48) found that potato Q-enzyme does not act on amylose chains less than 25 glucose units long. If it be accepted that Q is a transglycosidase, it must be one which shifts whole amylosyl groups rather than a single hexosyl group from a C-4 to a C-6 position (49). Nussenbaum & Hassid (50) have shown that the relatively short amylose chains (23 to 42 glucose units long) are unaffected by Q-enzyme, while chains averaging 116 units in length are converted into a branched product. In a recent study, furthermore, Bailey *et al.* (51) utilized maltohexaose as the phosphorylase primer and observed the action of Q-enzyme on the amylose synthesized by phosphorylase from glucose-1-phosphate and the maltohexaose at different stages of the reaction. An appreciable affect of Q was found only after chains 42 glucose units long had formed, the action of Q being 20 to 25 times more rapid with 58 unit amylose than with 30 unit amylose. Nussenbaum & Hassid (50) find that the branched product formed at successive stages of the reaction shows increasing degree of branching. The branched product could be precipitated with pentasol up to a fairly late reaction stage. They point out that this is in accord with the view that Q acts at random on all amylose molecules rather than repetitively on a single chain.

An elegant method for the crystallization of Q-enzyme was developed by Gilbert & Patrick (52) on the basis of fractionation with alcohol at low ionic strength and recrystallization from ammonium sulfate solutions. Purified enzyme obtained in this way was sensitive to ions and activated by ammonium.

A flagellated protozoa, *Polytomella coeca*, has been successfully utilized by Bebbington *et al.* (53) as a Q-enzyme source. The protozoan Q seems very similar in its properties to the potato Q-enzyme. That the product formed from potato amylose by the protozoan Q-enzyme is of the branched type has been shown unambiguously by the use of the methylation technique (54). It is interesting to note that the reaction of the protozoan Q-enzyme on

amylose shows an autocatalytic trend. As might be expected on the basis of this observation, the lag phase of the reaction was reduced considerably on the addition of glycogen or dextrins to the system (55).

Dextransucrase.—The potential importance of dextrans as plasma-volume expanders has evoked increased interest in the details of the structure of these polysaccharides and an attendant concern with the enzymes which bring about their synthesis and degradation. A timely study of cultural factors governing the elaboration of dextransucrase by *Leuconostoc mesenteroides* (NRR1 B-512) has been published by Tsuchiya *et al.* (56). The dextran formed by this organism is of the essentially linear type, with at least 95 per cent of α-1,6-glucosidic linkages. According to Stodola *et al.* (100), the utilization of sucrose by *L. mesenteroides* leads to the appearance of a new disaccharide, "leucrose," in the culture medium. The latter is assigned the structure 5-(D-glucopyranosyl)-D-fructopyranose. Its role in the synthesis of dextran remains to be clarified.

Dextranases.—Growth of suitable strains of *Penicillium* and *Aspergillus* on culture media containing linear-type dextran as carbon source resulted in appearance of considerable dextran-hydrolase activity in the extracellular phase both towards the linear and towards highly branched dextran types (57). The enzyme preparation from *Penicillium* formed isomaltose as the main reaction product (58). *Aspergillus* strains, on the other hand, yielded an enzyme preparation in which rapid breakdown of the isomaltose occurred (57, 59). Attention has been directed to the potential usefulness of mid-group types of dextranase in conversion of the native dextran into a polymer within the weight range required for use as a plasma-volume expander (60). The occurrence of dextranase in the supernatant fluid of cultures of anaerobic bacteria has been reported by Hehre & Sery (61).

SYNTHESIS AND CLEAVAGE OF FRUCTOSIDES

Fructosyl transfer reactions.—A transient formation of presumably glucose-terminated oligofructosides is observed when fructofuranosidases, e.g., yeast invertase preparations, act on sucrose (63, 64). The inference is acceptable that fructose transfer reactions are responsible for the production of the oligosaccharides. The question whether the transfer reaction leading to the formation of the oligosaccharides is mediated by sucrose hydrolase itself or by a specific transfer enzyme has not been solved. The claim of Aronoff (65) that a certain yeast invertase preparation fails to effect synthesis of the oligofructosides has been shown to be erroneous (66). White & Secor (72) have found that the oligosaccharides formed in the course of the reaction include a reducing disaccharide other than turanose. At least four oligosaccharides including two reducing sugars have so far been differentiated in the mixture of the reaction products (66). It would be important to ascertain whether the two reducing saccharides originate through transfer of glycosyl to a reducing acceptor sugar or by hydrolysis of higher oligosaccharides. In the case of the action of honey invertase on sucrose, six oligosaccharides appeared (66). It

seems probable that transglucosidification rather than transfructosidification is operative in the honey invertase system.

Dedonder (67) has shown that a *Bacillus subtilis* preparation forms several oligosaccharides as well as high-polymer levans from sucrose, and he has ascribed the production of all these saccharides to the activity of levansucrase. However, it is known that levansucrase in this preparation is associated with a sucrose hydrolase (68) and with a levan hydrolase (89), and it is conceivable that hydrolase activities rather than levansucrase account for the production of the lower homologues.

The formation of lower members of the inulin series from sucrose by the action of extract of tubers of Jerusalem artichoke has been studied in further detail by Dedonder (69). In agreement with the work of Edelman & Bacon (71), it is suggested that the enzyme inulosucrase is analogous to levansucrase in its reaction pattern. Inulosucrase is presumed to mediate growth of sucrose-terminated fructosyl chains by repetitive transfer of single fructosyl residues from sucrose to the fructosyl terminal of the sucrose-terminated fructosyl chain. While the evidence is impressive that the inulin polymers form a sucrose-terminated fructosyl chain homologue series, it should be noted that acceptance of this concept has not been unanimous [Schlubach *et al.* (70)]. Dedonder considers that sucrose itself may serve as a fructosyl acceptor. Edelman & Bacon (71), on the other hand, have stated that sucrose fails to donate fructose to sucrose to an appreciable extent. In accord with expectation, the reaction mediated by inulosucrase in the tuber extract of Jerusalem artichoke showed no dependence on inorganic orthophosphate. Curiously enough, however, inorganic orthophosphate proved to be necessary for the mediation of the same reaction by the stem extract of Jerusalem artichoke (69).

Hydrolytic reactions.—The action of yeast invertase on the lower members of the inulin homologue series leads first to liberation of fructose and to delayed appearance of free glucose [Dedonder (73)]. With an inulase preparation of *Sterigmatocystis nigra*, furthermore, a transient appearance of free sucrose could be detected as well. The findings can thus be summarized in the following reaction scheme:

$$\begin{aligned}&\text{D}+\text{water}\rightarrow\text{C}+\text{fructose}\\&\text{C}+\text{water}\rightarrow\text{B}+\text{fructose}\\&\text{B}+\text{water}\rightarrow\text{sucrose}+\text{fructose}\\&\text{sucrose}+\text{water}\rightarrow\text{glucose}+\text{fructose}\end{aligned}$$

where B, C, D represent sucrose-terminated fructosyl chains of increasing polymerization degree.

The influence of the polymerization degree on the cleavability of glucofructosans by yeast invertase has not been determined in a precise way. That the hydrolysis of the higher members of the inulin homologue series by yeast invertase preparations is mediated by a specific polyfructosidase rather than by sucrose hydrolase is supported by the observations of Legrand & Lewis (74). They found that the dialyzate of autolyzate of baker's yeast hydrolyzes

glucofructosans (e.g., asphodeloside of *Narcissus pseudo-narcissus*) and not sucrose, whereas the residual nondialyzable fraction hydrolyzes sucrose and not the glucofructosan. As has been shown by Edelman & Bacon (75), hydrolysis of inulin by the extracts of Jerusalem artichoke may occur in the absence of sucrase activity.

The hydrolysis of the trisaccharide melezitose by an enzyme of *Proteus vulgaris* OX 2 has been the subject of an elegant study by Hehre & Carlson (76). They found that the *Proteus* melezitase acted preferentially on the acid-stable linkage of melezitose, and they isolated sucrose from the reaction mixture. There can remain little doubt that melezitose is in fact a sucrose derivative, 3-[α-D-glucopyranosyl]-β-D-fructofuranosyl α-D-glucopyranoside.

An interesting study of the purification of yeast invertase has been contributed by Fischer *et al.* (77, 78). The purified yeast invertase protein was found to be stabilized by an accompanying yeast polymannan. When the latter was separated from the yeast invertase by treatment with bentonite, the invertase thus purified became highly labile and rapidly lost its activity. The condition of invertase in yeast, according to Thorsen & Myrback (79), is that of an insoluble complex. The complex can be resolved by proteolytic enzymes of the papain type, and it is suggested accordingly that invertase in the native state comprises a protein component which may be split away without loss of activity.

Notwithstanding the fact that the kinetics of invertase action have been studied carefully by many schools of investigators, the nature of the elementary reaction steps of sucrose hydrolysis by invertase remains as mysterious as ever. Myrback & Willstaedt (80) have observed that the pH activity curve of invertase is shifted in the acid direction on the acid side of the pH optimum when the substrate concentration is increased. They suggest in their explanation of this finding that a charged $—NH_3^+$ group on the active surface of the enzyme, unlike the uncharged $—NH_2$ group from which this charged group arose, fails to combine with the substrate.

The energetics of the hydrolysis of the acetal linkage of sucrose by invertase have been studied recently by Bauer & Gemmill (81) who have reported that the heat production of the enzyme-mediated reaction is 3.28 kcal./mole as compared with 3.563 kcal./mole for acid hydrolysis of the same linkage. The dimensions of invertase, as determined by Pollard *et al.* (88) with use of fast electrons and deuterons as a probe, are 48 by 83 angstroms, and the molecular weight computed on this basis is thought to be 123,000.

SYNTHESIS AND CLEAVAGE OF β-GLUCOSIDES

Glycosyl transfer reactions.—Rabaté (83) first showed that plant β-glucosidase preparations may catalyze a transfer of the β-glucosyl group, but many years elapsed before the fundamental nature of this discovery was realized. More recently, Miwa *et al.* (84) and Tabano & Miwa (85) have demonstrated that the β-glucosyl group of *p*-nitrophenyl-β-D-glucoside and β-phenylglucoside may be transferred to methanol, ethanol, or butanol by

β-glucosidase preparations from a variety of plant and fungal sources. The findings were open to the interpretation that the same enzyme entity may mediate transfer of the β-glucosyl group to alcohols and to water rsepectively. Critical tests of this point will be of great interest.

The occurrence of oligogalactosides as transient reaction products of the process of lactose hydrolysis has been described by Aronson (82). When lactase derived from *Saccharomyces fragilis* acted on lactose, at least four distinct oligosaccharides could be resolved by paper chromatography: lactose, a galactoglucose other than lactose, a digalactoside, and a digalacto-glucose. In addition to lactose, other compounds (glucose, galactose, xylose, glycerol) served this enzyme as acceptors of the galactosyl group. A similar transfer phenomenon was also observed with lactase of *Escherichia coli*. Aronson showed that the formation of oligosaccharides from lactose is not unique to the case of enzyme-catalyzed hydrolyses but also occurs when the lactose is hydrolyzed by a mineral acid.

An instance of transglycosidification which may well play a role of great biological importance is seen in the action of an enzyme system from *Lacto-bacillus helveticus* on deoxyribosyl nucleosides [Macnutt (86)]. The enzyme, which was designated trans-N-glycosidase, transfers the deoxyribosyl group from a purine or pyrimidine nucleoside to another purine or pyrimidine. Unlike the known sucrose transglucosidase, the trans-N-glycosidase is unable to use inorganic phosphate as acceptor and fails to act with deoxyribosyl-1-phosphate. Kalckar, Macnutt & Hoff-Jorgensen (87) utilized C^{14} labelled adenine to examine the mechanism of the action of the enzyme. Since,

$$[C^{14}]\text{-adenine} + \text{hypoxanthine deoxyriboside} \rightleftharpoons \text{hypoxanthine} + [C^{14}]-\text{adenine deoxyriboside}$$

and,

$$\text{adenine deoxyriboside} + [C^{14}]-\text{adenine} \rightleftharpoons [C^{14}]-\text{adenine deoxyriboside} + \text{adenine}$$

it followed that the enzyme catalyzes a transglycosidification rather than a transamination.

Histochemical test for glycosidases.—An ingenious histochemical method for the detection of glycosidases has been developed by Seligman and co-workers on the basis of the use of brominated naphthylglycosides as test substrates (90, 91, 97). The insoluble bromo-naphthyl alcohol liberated by hydrolysis is visualized by coupling with tetrazotized diorthoanisidine.

Biosynthesis of β-galactosidase.—The thought provoking researches of Lederberg (92) and of Cohn & Monod (93) on the mode of the induction of β-galactosidase activity in *E. coli* posed the question whether the synthesis in response to the carbohydrate inducer represents a process which requires the presence of the specific inducer, or an enhancement of a process which also occurs, though to a lesser degree, in the absence of the specific inducer. Leser & Bonner (94) have observed that widely different carbohydrate inducers establish widely different levels of β-galactosidase activity in the cells. Whereas lyxose and raffinose were not themselves utilized by the cells

for growth, they stimulated enzyme synthesis when maltose was provided as a carbohydrate energy source. A similar situation had previously been shown for the case of melibiose as the inducer and maltose as the energy source by Cohn & Monod (93). Lester & Bonner (94) conclude in the light of their results that the inducers govern quantitative changes in a normal physiological process and that they do not generate any reaction *de novo*.

Cohn & Torriani (95) have conducted a survey of the occurrence of the P_z protein, an immunological relative of the β-galactosidase protein, in species of *Enterobacteriaceae*. The survey revealed no instance in which synthesis of β-galactosidase occurred without presence of P_z protein in the cells. P_z is thought to be either a precursor of the enzyme or a product whose formation is dependent on the same genetic and extrinsic factors which govern the synthesis of the β-galactosidase (cf. 96).

Laminarinase.—The cleavage and synthesis of β-linkage polyglucosides had remained a relatively neglected field for many years. The work of Dillon & O'Colla (98, 99) on the hydrolysis of a group of β-C-1: C-3 polyglucosides is therefore particularly timely. They find glucose as the product of the hydrolysis of lichenin; glucose and laminaribiose as products of the hydrolysis of yeast glucan; and glucose and a number of oligoglucosides as the products of the hydrolysis of laminarin by extracts of plants (oats, barley, potato, and hyacinth tubers). It cannot be stated whether these reactions are mediated by one or by several enzymes. Analogy with the starch field would suggest, however, that a number of enzymes may be concerned.

GLYCOSIDASES ACTING ON SACCHARIDES CONTAINING AMINO OR CARBOXYL GROUPS

The rapid advance made in the study of the degradation and synthesis of amylosaccharides and fructosides has not been accompanied by a comparable advance in our knowledge of the synthesis and degradation of the important group of the saccharides which contain polar groups. Work in this field has remained restricted to consideration of hydrolysis alone, and nothing is as yet known concerning the modes of synthesis of these compounds.

β-Glucuronidase.—β-glucuronidase may be specifically involved in the metabolism and action of estrogenic hormones (101), and the elucidation of the factors which govern the *in vivo* activity of this enzyme was therefore of particular interest. Walker (102) finds that the β-glucuronidase activity of mouse liver is associated with subcellular particles and that the enzyme may be liberated partially or completely from the latter by treatment with hypotonic solutions. The full activity of a homogenate was only evinced after at least one half of the total enzyme amount had been removed from the association with the particles. In rat liver homogenates, the assay of the enzyme was complicated by the presence of a thermostable nondialyzable inhibitor (103). The effect of the latter could be countered by addition of a suitable surface-active agent (Triton X-100) to the homogenate system.

It had been suggested that the coenzyme of β-glucuronidase is a deoxy-

ribonucleic acid (104, 117). However, Smith & Mills (105) have criticized this conclusion and have pointed out that the activating effect of nucleic acid might be a particular instance of the activation of β-glucuronidase systems by acidic polyelectrolytes.

β-glucuronidase was separated in a cell-free state from medium-sized microorganisms of the sheep rumen by Karunairatnam & Levvy (106). Thei experiments describing the general properties of the enzyme were conducted on phenolphthalein-β-glucuronide as substrate. The inhibition of the activity by substrate excess was in agreement with the view that the enzyme forms an inactive complex with two molecules of the substrate.

Methods for the histochemical localization of β-glucuronidase have been based on the use of the glucuronide of 1- or 2-naphthol, 6-bromo-2-naphthol, 1-(O-hydroxy phenylazo)-2-naphthol, and 8-hydroxyquinoline as the enzyme substrate [Friedenwald & Becker (119); Seligman (120); Seligman & Rutenberg (121)]. The comparative study by Burton & Pearse (118) has suggested that 8-hydroxyquinoline glucuronide is the best of these, and that its use with a modified form of the procedure proposed by Friedenwald & Becker is relatively free from the theoretical and practical objections which vitiate the histochemical application of the remaining above-listed substrates of β-glucuronidase.

Pectinases.—A critical analysis of the literature up to 1950 has been contributed by Kertesz (107). The recent recognition that there are several enzymes mediating the cleavage of the glycosidic linkages of pectins with characteristically different action patterns promises to bring more order into this confused field.

It had been assumed that the hydrolysis of the interglycosidic linkages in pectin was necessarily preceded by esterase action. It has become evident, however, that specific glycosidases may act selectively either on the esterified or on the nonesterified pectin polymers. Seegmiller & Jansen (108) have shown that a glycosidase (polymethylgalacturonase) from a commercial enzyme source ("Hydralase") directly catalyzes the hydrolysis of the esterified pectin (polymethylgalacturonic acid), since it acts much more rapidly on the esterified than on the deesterified polymer. This enzyme converted the polymethylgalacturonic acid into a mixture of polymeric products. The reaction proceeded to an arrest point at circa 26 per cent of total hydrolysis, and no monogalacturonic acid or methylgalacturonate were set free by this degradation. Roboz, Barrat & Tatum (109) described an extracellular enzyme from *Neurospora crassa* which mediates a similar degradation process. The molecular weight of the polyuronide reaction product was estimated on the basis of electrophoretic behaviour to be about 4000.

A chromatographic analysis of the degradation products formed from pectin by fungal pectinase has suggested that mono-, di-, tri-, tetra-, and penta-saccharides occur in the mixture of reaction products [Jerwin & Tomkins (116)]. Some of the intermediary reaction products formed when the hydrolysis of pectic acid is mediated by the polygalacturonase system of

Aspergillus species have been resolved with the aid of paper chromatography by Altermatt & Deuel (110). Mono-, di-, tri-, tetra-, penta-, and higher galacturonic acid homologues could be identified among the products of the reaction, the larger homologues progressively giving way to the smaller as the reaction progressed. These results are in agreement with the supposition that the linkages in the polygalacturonic acid chain are hydrolyzed in a random manner. However, the possibility that more than one enzyme may have been involved in the process should not be excluded. Schubert (111) has presented evidence in support of the conclusion that four distinct glycosidase entities with activity towards pectins may be present together in *Aspergillus niger*.

The claim that the effect of *Byssochlamys fulva* on pectin is solely mediated by a polymethylgalacturonase enzyme has been studied by Reid (112). He finds, however, that this fungus produces a pectin esterase as well as a polygalacturonase.

The hydrolytic action of yeast polygalacturonase on pectic acid has been analyzed by Phaff & Luh (113, 114). With purified enzyme, they found that the reaction proceeds to an arrest point when the hydrolysis approaches 50 per cent of the theoretical value for the total degradation. At this stage about 16 per cent of the substrate had been converted to monogalacturonic acid. The remaining free acids were resolved by chromatographic analysis into two components. These were purified and isolated and shown to be digalacturonic and trigalacturonic acid respectively. The findings seem in accord with the view that yeast polygalacturonase can only attack the polygalacturonic acid chain if it is at least four galacturonic acid units long. Fungal polygalacturonase, on the other hand, converts the oligogalacturonic acids into monogalacturonic acid. Conceivably, the chain length specificity of the fungal enzyme is broad as compared to that of the yeast enzyme, or the fungal system contains a special oligogalacturonase in addition to the polygalacturonase.

Hyaluronidases.—In the absence of definitive clarification of the structure of hyaluronic acid, interpretations of the enzyme-catalyzed reactions shown by this substrate are necessarily of a tentative nature. During the period under review, an important advance towards a better understanding of the hyaluronidase systems has been signalled in the work of the group at Columbia led by Karl Meyer. A good review of the chemical and biological aspects of hyaluronidase action has recently been published (122).

Study of the degradation of hyaluronic acid by a hyaluronidase preparation from *Pneumococcus*, type II necessitated the revision of an earlier conclusion that this enzyme liberates monosaccharides (123). It was confirmed that the products of hyaluronidase action exhibit unusual reducing values in the standard methods of sugar analysis (cf., also 124), and that confusion had arisen in the literature from failure to consider this point. The analysis of the reaction products of hyaluronidase action with the aid of a flow column technique (125, 126) showed that no monosaccharides are formed when the

pneumococcal enzyme or purified testicular enzyme acts on hyaluronate (123). The properties of the end-product of *Pneumococcus* enzyme action were consistent with the hypothesis that this is a disaccharide or a mixture of disaccharides composed of one residue of glucuronic acid and one of N-acetylglucosamine, with the free reducing group in the latter. Properties of this product, notably the ratio of its reducing value to its acetylglucosamine equivalent and the flow behavior on the column differed clearly from those of the end-product of the action of purified testicular enzyme on hyaluronate. Moreover, pneumococcal enzyme caused a rapid liberation of reducing sugar when added to the product of the prolonged action of purified testicular enzyme on hyaluronate. When intermediary products of the reaction mediated by pneumococcal enzyme were assayed, a constant ratio value was found for reducing value: acetylglucosamine equivalent. This is cited in support of the conclusion that the enzyme acts in a manner similar to that of testicular hyaluronidase by hydrolyzing only N-acetylglucosaminidic bonds.

In their parallel study of the action of purified testicular enzyme on hyaluronate, Rapport, Meyer & Linker (126) confirmed the earlier finding by Hahn (124, 127) that monosaccharides do not occur among the reaction products. The chromatographic analysis revealed that a large number of molecular species occur as intermediary products of the reaction. The enzyme thus probably acts as a mid-group enzyme, rather than at a fixed distance from the chain ends. All the fractions obtained contained equimolar proportions of glucosamine and uronic acid. Hence it seems unlikely that hyaluronate contains a chitin-like core of N-acetylaminosugar units [Kaye & Stacey (144)], and the view is supported rather that the polymer consists of alternating N-acetyl glucosamine and glucuronic acid residues. Even the end-product of the action of the testicular enzyme is clearly a mixture. The reducing value and the behaviour with *Pneumococcus* enzyme accord with the assumption that the end-product in this case is predominantly a tetrasaccharide.

The production of monosaccharides from hyaluronate by crude hyaluronidase preparations had been ascribed by Hahn to a specific oligosaccharase acting on the products of the action of the true hyaluronidase. Since testicular hyaluronidase preparation contains β-glucuronidase activity, the possibility required consideration that the β-glucuronidase is responsible for the monosaccharide formation. Meyer, Linker, & Rapport (128) confirmed this assumption. They found that a product of the action of purified hyaluronidase is hydrolyzed by the crude system as well as by purified liver β-glucuronidase with liberation of monosaccharides. It should be noted, however, that the disaccharidic fraction which is the end-product of the action of *Pneumococcus* enzyme on hyaluronate proved resistant to the action of the β-glucuronidase.

Mathews *et al.* (129) have found that the same testicular enzyme is involved in the depolymerization of chondroitin sulfuric acid and hyaluronic acid. Both activities are inhibited by the hyaluronidase serum inhibitor; the

ratio of sulfomucase to hyaluronidase activity remained constant for a number of enzyme fractions of differing purity. It is proposed that the hyaluronidase unit already in use should also be applied to sulfomucase. This work is in disagreement with that of Lillie *et al.* (130) who based their studies in part on histochemical procedures.

Methods for measuring hyaluronidase activity still contain uncontrolled factors, in large part owing to variability in substrates. Alburn & Whitley (131) have studied a variety of factors affecting the turbidimetric method of hyaluronidase assay. A definite relationship between pH and NaCl concentration was found. Loss of enzyme activity during assay could be avoided by addition of gelatin as a stabilizer. The range limits of pH, ionic strength, temperature, and enzyme concentration within which purified testicular bovine hyaluronidase preserves activity have been described more closely in a useful study of Freeman & Webster (132). The turbidimetric assay method has been examined by Bachtold & Gebhardt (133). An analytical approach based on the binding of bromosulfalein by a hyaluronate-protein complex has been explored by Grief (134). An interesting assay based on titration of bull testis hyaluronidase potency with rabbit antiserum has been utilized by Evans *et al.* (135).

Increasing attention has been given to some naturally occurring hyaluronidase inhibitors. Wattenberg & Glick (136) noted a 60-fold purification of the "nonspecific" inhibitor present in blood on precipitation with protamine sulfate, and a 130-fold purification on precipitation at pH 5.4 and alcohol fractionation. The purified material showed a marked enrichment of metachromatic staining capacity. It is noted that these properties are in accord with the view that the inhibitor is an acidic polysaccharide, e.g., a heparin, and that it acts as a substrate analogue. Within tissue rich in mast cells, Glick & Sylven found a heparin-lipoprotein which inhibits hyaluronidase (137). The relationship of this factor with the serum inhibitor remains to be clarified. Furthermore, Gibian (138) working with dog saliva found that this fluid too contains a hyaluronidase inhibitor. The latter was found active at neutral, but inactive at acid pH. The activation of serum inhibitor by Mg^{++} and Co^{++} ions has been reported by Mathews *et al.* (139). The activating effect of cobalt ion was found to be exhibited also by its complex $[CO(NH_3)_5Cl]^{++}$, and the authors suggest accordingly that the activating effect may be mediated by interionic forces which extend beyond the relatively stable coordination sphere. An adreno-cortical control of the inhibitory activity of serum on hyaluronidase has been suggested by the study of Good *et al.* (140) who have found that the serum inhibitory activity increases under conditions of stress, but only when the adrenals are intact. In this connection it may be of interest that cortisone and deoxycortisone acetate have been found to be inhibitors of hyaluronidase at physiological concentrations (141).

A systematic study of the effect of a collection of low molecular and highpolymer substances on hyaluronidase by Pantlitschko & Kaiser (142) has

suggested some generalizations concerning the properties necessary for hyaluronidase activation and inhibition. Activation is observed with positively charged polymers, e.g., histone, protamine, peptone. Their effect is countered by negatively charged polymers. On the other hand, inhibition is observed with strongly negatively charged polymers provided that their structure is fibrous. Thus sulfate esters of cellulose, carboxycellulose, and hyaluronic acid proved inhibitory, whereas starch, glycogen, sulfate esters of starch and glycogen, carboxycellulose, and its monosulfate lacked this activity. Metallic cations (ferric, cupric, ferrous, and zinc) have been found to exert a reversible inhibitory action, that of Fe^{+++} being best reversed by pyrophosphate, whereas that of Cu^{++} was most effectively reversed by cysteine [Meyer & Rapport (143)].

Lysozyme and other mucases acting on components of cell surfaces.—In the prevailing state of our ignorance concerning the chemical nature of the mucopolysaccharide components of cell surfaces, the sparsity of our knowledge concerning the chemical basis of the profound changes undergone by the cell surface in the presence of specific enzymes occasions little surprise.

A fundamental enquiry into the reactions of the surface of the red cell in the presence of the enzyme of influenza virus has been undertaken by Gottschalk (145). The work is premised on the assumption that mucins which act as inhibitors of influenza virus represent analogues of the substrate present in the red cell surface. Study of the action of purified influenza virus on such mucins could therefore be expected to throw light on an important physiological function of the viral enzyme [cf. Burnet (146)]. The inactivating action of highly purified influenza virus on an electrophoretically homogeneous preparation of the human urine mucoprotein inhibitor of virus haemagglutination was found by Gottschalk (145) to be attended by the liberation of a dialyzable nitrogen-containing carbohydrate. The properties of this compound were in accord with the notion that it represents an amino acid (or peptide) linked by way of the amino-group to an isoglucosamine (fructosamine). The ratio, haemagglutinin titre: enzymic activity, of the influenza virus was not significantly changed by procedures of virus purification in the course of which up to 99 per cent of the nitrogen of the crude virus preparation was removed [Gottschalk & Perry (147)]. This result has strengthened the assumption that the enzyme activity is an integral part of the virus particle and not a contaminant of the latter.

Several laboratories have carried forward the analysis of the structure of the lysozyme molecule. A nearly complete balance sheet of the amino acid content of crystalline lysozyme has been accomplished [Fromageot *et al.* (148, 149); Lewis *et al.* (150)]. Evidence drawn from this source was consistent with the view that the lysozyme particle contains less than seven and probably only one or two peptide chains (150). More recently, the elucidation of the sequence in which the amino acids occur has come under consideration. Acher, Justisz & Fromageot (151) have presented analyses of basic peptide fractions obtained from lysozyme by partial acid hydrolysis. Green &

Schroeder (152) have isolated α,ϵ-dinitrophenyl-lysine from the ether extract of an acid hydrolyzate of dinitrophenyl-lysozyme and have inferred that not more than a single lysine residue occurs at an amino terminal position in lysozyme. From the analysis of the partial hydrolyzate of dinitrophenyl-lysozyme, Schroeder (153) has deduced that the sequence of four amino acids at the amino end of the peptide chain is lysyl-valyl-phenyl-phenylalanyl-glycyl.

Information concerning the internal structure of the lysozyme peptide chain has been sought by Fraenkel-Conrat *et al.* (154) through the study of the effect of reduction of lysozyme by thioglycol. In presence of urea at pH 5, reduction of the molecule by thioglycol, once started, appeared to proceed rapidly to completion, and afforded a product which could be quantitatively alkylated with iodoacetamide at pH 7 to 8.5 and thereby rendered insoluble. The molecular weight of the reduced and alkylated lysozyme, as determined by osmotic pressure measurements, did not differ from that of the original protein. This is cited as supporting the conclusion that lysozyme consists of a single peptide chain, internally crosslinked by disulfide bonds.

An interesting reaction which is induced in lysozyme carbonate by recrystallization has been discovered by Tallan & Stein (155). They have shown that the directly crystallized lysozyme carbonate, which is chromatographically over 95 per cent homogeneous, is converted by repeated recrystallization into two active components. These may be separated chromatographically. The formation of insoluble complexes between lysozyme and anionic and cationic detergents has been reported by Glassman *et al.* (156).

It seems desirable to point out that the attractiveness of lysozyme as a model for research on protein structure would be enhanced by more definite information concerning the chemical reaction catalyzed by this relatively simple and readily purified enzyme molecule. The existing fragmentary information concerning the chemical function of lysozyme has been reviewed by Fishman (157). Holyoke & Johnson (158) have found that an increase in the rate of the lysozyme reaction is produced by an elevation of the hydrostatic pressure. Recently, Salton has reported the preparation of the cell wall material of *Micrococcus lysodeikticus* in the form of an opalescent suspension, and has shown that lysozyme effects a progressive and almost total disintegration of the suspended cell-wall particles (159).

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CHEMISTRY OF THE CARBOHYDRATES[1]

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Only a small part of the research in carbohydrate chemistry reported in the last year can be covered in the present review, but fortunately, certain phases of the subject are treated in the chapters devoted to biological oxidations, carbohydrate metabolism, and immunological polysaccharides. Recent progress in carbohydrate chemistry relating to enzymes (1) nucleic acids (2), and carbohydrate metabolism (3) were lucidly and thoroughly covered in the 1952 *Annual Review of Biochemistry.* Special aspects of carbohydrate chemistry are to be found in other reviews and bibliographies (4 to 8).

Research on carbohydrates and their roles in complex biological systems has recently shown phenomenal progress, in large part, as a result of the impetus gained from new techniques of separation and analysis, from certain new concepts, and from the application of radioactive tracer methods.

Methods for the Separation of Carbohydrates and Carbohydrate Derivatives

Paper chromatography.—Utilization of the techniques of chromatography (9, 10) has made possible the isolation and study of carbohydrate constituents of perhaps hundreds of biological systems. For the detection and isolation of carbohydrates in minute quantities, paper chromatography is ordinarily the most suitable. The appearance of a concise and practicable laboratory manual on the subject (11) will relieve many workers of an extensive literature search for suitable techniques. A number of spray reagents for detection of sugars in paper chromatography are described in this manual, and others were mentioned in the preceding review (12). Interest in new color reagents and in application of old ones continues, with emphasis on differentiation of one carbohydrate, or group, from another. For general detection of sugars, aniline phosphate in butanol (13) appears to show a wider range of reaction than aniline hydrogen phthalate. Hexoses, pentoses, and certain other sugars have been differentiated on paper by *p*-anisidine phosphate in ethanol (14). Diethyl-*p*-phenylenediamine sulfite has been found to distinguish between melibiose and levulosans. A method (15) for identifying raffinose in the presence of fructosyl-sucrose [kestose (16, 17, 18)] makes use of this reagent, in conjunction with hydrolysis on paper by invertase (19). For the determination of raffinose in beet molasses the 1-naphthol-ethyl alcohol-

[1] The survey of the literature pertaining to this review was concluded in December, 1952.

[2] The authors are grateful to Dr. R. Schaffer for generous assistance in the literature search for this review.

phosphoric acid reagent (20) was found to be most suitable (21). Utilization of an orcinol-trichloroacetic acid spray for the differentiation of ketoheptoses and other ketoses (22, 23) has facilitated the identification of sedoheptose in numerous plant extracts (24, 25, 26).

By successive developments, with intermediate drying of the paper (multiple development), it was found possible to increase the separation of slow moving saccharides (27). A quantitative adaptation of the method, in which resolved carbohydrates are eluted and determined by anthrone reagent, provides a satisfactory method for the determination of the saccharides present in complex mixtures (28).

Carbon chromatography.—Whereas paper chromatography is an elegant method for the separation of microgram quantities [11 (p. 78)] it, nevertheless, can be so conducted as to obtain amounts of material of the order of 100 mg. from a single chromatogram (29). Of more general usefulness, however, for large-scale separation of monosaccharides and various disaccharides is the carbon chromatography method of Whistler & Durso (30) in which adsorbed saccharides are separated by elution, first with water, and then with increasing concentrations of ethanol. By this procedure two crystalline disaccharides (31) and a crystalline trisaccharide, α-D-galactopyranosyl-(1→6)-β-D-mannopyranosyl-(1→4)-β-D-mannopyranose (32) have been separated from hydrolyzates of guaran. From the structural data it was concluded that the guaran polysaccharide consists of a chain of mannopyranose units linked β-1→4 and that on the average a D-galactopyranose unit is linked α-1→6 to every other D-mannose unit. The results obtained from the hydrolyzate of xylan were even more striking; the entire series of saccharides up to and including the hexasaccharide were separated by the carbon chromatography technique and ultimately crystallized. These represent the first crystalline oligosaccharides composed entirely of pentose units. They differ from the oligosaccharides derived from cellulose in that they lack a projecting CH_2OH group on each ring (33). Many applications of the Whistler-Durso technique have been made; typical of these is the separation of biosynthetically prepared C^{14}-labeled carbohydrates (34).

Chromatography using ion-exchange resins.—Although carbon chromatography effects a separation of saccharides into groups of similar size and structure, it is less effective in the separation of the constituents of a single group. For substances possessing acid groups, such as the closely related sugar phosphates and nucleotides, much success has been achieved in separation by adsorption on ion exchange resins, usually in the salt form, and elution with solvents of suitably adjusted pH (35, 36, 37). The separation of glucuronic and galacturonic acids can most readily be made by adsorption on the acetate or formate form of a strong base anion exchanger, and elution, respectively, with 0.15 *M* acetic acid or 0.01 *M* formic acid (38). Separation of many sugars can be effected by virtue of their capacity to form complex ions with borates (39, 40). The sugars, or other diol-containing substances, are adsorbed on borate-treated anion exchange resin and eluted with boric-

borate buffers (41). Sharp separations are obtained among the individual members of a group of closely related saccharides. The method has been shown to be applicable to nearly all of the naturally occurring sugars and sugar alcohols, and a procedure for the removal of the borate from the isolated complex makes this method suitable for preparative purposes (42). Separation of glucosides, however, is not too successful because of the slight tendency of the glucopyranoside structure to form a highly ionized borate complex (43). Combination of the borate-complex adsorption technique with elution by buffers progressively adjusted as to pH, and ionic character has permitted the quantitative separation and recovery of each component from a mixture of glucose, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, ribose-5-phosphate, fructose-1,6-diphosphate, 2-phosphoglyceric acid, adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate, [(44); see also (45, 46)]. Borate complexes, by imparting ionic mobility, have also facilitated separation of sugars (47, 48) and of nucleosides (49) by paper electrophoresis.

Application of Carbon-14 Tracer Techniques

A review of current chemical and biological literature cannot fail to impress the reader with the increase in the application of radioactive tracer techniques to the various phases of carbohydrate chemistry. The versatility of carbon-14, particularly, is shown by the fact that there is hardly a field in biochemistry in which some application of this new tool has not already been made.

Analytical procedures.—Use of carbon-14 does not involve an appreciable radiation hazard (50) and does not require elaborate equipment. There are several reliable counting instruments suitable for assay of carbon-14, which can be purchased for less than $1000. With a proportional counter, material can be assayed directly in the form of thin films or in formamide solution. The formamide method (51), although extremely simple, requires relatively large samples (about 0.1 μc. per ml. of formamide), whereas films for counting require about 0.0002 μc. in less than 2.5 mg. of material (52). By use of a vibrating reed electrometer 0.0001 μc. of carbon-14 in 1 millimole of CO_2 is readily measured. This quantity of carbon would be contained in 1×10^{-6} mg. of commercial sodium cyanide-C^{14} having an activity of 5 mc. per millimole. The extreme sensitivity of radioactivity measurements and the facility with which routine analyses can be made, make possible a wide variety of analytical and research techniques. Thus, reducing end groups in polysaccharides can be estimated with C^{14}-labeled cyanide (53) and minute quantities of free sugars can be determined from the amount of C^{14}-labeled cyanide converted to nonvolatile compounds (54). For the determination of small quantities of specific sugars in complex mixtures, either the isotope dilution technique (55) or the reverse isotope dilution technique (56) is useful. In the former procedure one adds a tracer to the material to be analyzed and estimates the amount of the constituent from the activity of the product. In the latter or

reverse procedure one determines the amount of a radioactive compound formed, after addition of the nonradioactive counterpart. Thus if sugar A, present in minute quantity, reacts with radioactive reagent B*, the radioactive product AB* is separated by co-crystallization with a known quantity of nonradioactive AB. The amount of AB* formed can be calculated from the activity of the reagent, and of the mixed product.

Use of C^{14}-labeled carbohydrates.—Although the labeled carbohydrates have some use in theoretical organic chemistry (e.g., in the study of rearrangement reactions), their primary and unique value lies in the study of complex biological mixtures. Many of the striking results obtained by application of the labeled sugars belong properly in discussions of enzyme chemistry, metabolism, or photosynthesis. However, brief mention of a few results obtained in these fields will illustrate the usefulness of the C^{14}-labeled sugars. When rabbits were fed menthol and glucose-1-C^{14}, the isolated glucuronic acid was labeled predominantly in the C-1 position. It was concluded that glucose or an equivalent 6-carbon unit was the immediate precursor of glucuonic acid in the rabbit [(57); see also (58)]. On the other hand, evidence gathered from the administration of position-labeled lactate and pyruvate showed that these acids are incorporated into the glucuronic acid isolated from α-naphthol-fed rats (59). Lactose samples produced by lactating rats fed C^{14}-labeled starch (60), by lactating goats injected with glucose-C^{14} (61), and by slices of rat mammary gland incubated with glucose-C^{14} (62) were found to have equal distribution of carbon-14 in the glucose and galactose moieties of the molecules. A homogenate of guinea pig mammary gland capable of lactose synthesis from glucose-C^{14} has been prepared (63), but the mechanism of lactose formation is obscure. In all studies mentioned, galactose appears to be derived from glucose without passing through a 3-carbon intermediate. The carbon-14 distribution in the cellular polysaccharides of a yeast grown on glucose-1-C^{14} and acetate-1-C^{13} showed that mannose is derived directly from glucose rather than by synthesis from smaller units (64). The reverse process, conversion of mannose-1-C^{14} to glucose-1-C^{14} (as liver or muscle glycogen), was found to take place in the rat largely without cleavage of the carbon chain (65). Bacterial cellulose formed from D-glucose-1-C^{14} by *Acetobacter xylinum* was found to be labeled predominantly in the C-1 position of the glucose unit (66). Introduction of D-glucose-1-C^{14} into cotton bolls during the period of rapid cellulose formation gave approximately 50 per cent fixation of the carbon-14, with all of the activity in the first carbon of the glucose units (67). This technique makes possible the production of cellulose labeled at specific points of the glucose unit. The availability of position-labeled cellulose should stimulate investigation of the enzymic reactions involved in the synthesis and degradation of cellulose. Many studies of bacterial metabolism and fermentation processes have been made with position-labeled sugars. In a typical example (68), it was found by use of glucose-1-C^{14} and glucose-3,4-C^{14} that *Leuconostoc mesenteroides* converts carbon 1 of the sugar to carbon dioxide, carbons 2 and 3 to ethanol, and carbons

4, 5, and 6 to lactic acid. Use of radioactive carbon dioxide permitted discrimination between the initial products of photosynthesis and the components of tissues already present, and revealed that known intermediates of glycolysis are the principal products of short term photosynthesis (69). Sedoheptulose phosphate, which was also found to be among the early products of photosynthesis (70), has now been identified as a product of pentose phosphate metabolism with enzymes of animal origin (71).

Synthesis of labeled sugars.—Improvements have been made in the biosynthesis of the more common radioactive sugars (34, 72, 73, 74) and in the production of position-labeled sugars (75). Photosynthetically prepared, randomly-labeled glucose, fructose, and sucrose, as well as numerous chemically prepared, position-labeled sugars have now become commercially available. By various improvements in the cyanohydrin synthesis, the radiochemical yields of some of the position-labeled sugars have been raised to about 50 per cent. The existence of an acid-catalyzed mechanism in the addition of cyanide to aldoses has been recognized, and it has been found that in some cases the acid-catalyzed reaction gives a different proportion of epimers than the base-catalyzed (56, 76). For example, the base-catalyzed addition of cyanide to D-arabinose gives predominantly the gluconic epimer, but the acid-catalyzed addition gives the mannonic. By selection of suitable conditions for the condensation reaction, it is possible to obtain the maximum proportion of either epimer (77). Better control of the acidity in the sodium amalgam reduction of sugar lactones was accomplished by use of crystalline sodium acid oxalate as an internal neutralizing agent in place of the conventional adjustment by the addition of sulfuric acid (78, 79). As a result of this close control, it was possible to reduce as little as 18 mg. of gluconic-γ-lactone to glucose with a yield by analysis of 85 per cent (80). By the modified cyanohydrin method, 1-C^{14}-labeled glucose and mannose (78), lactose (56), galactose [(81); see also (82)], arabinose, and ribose (79) have been prepared in good yield. Application of the cyanohydrin synthesis to D-arabinose-1-C^{14} has yielded D-glucose-2-C^{14} (83). L-Arabinose-1-C^{14} has also been synthesized by the nitromethane method (84).

A unique conversion of D-glucose-1-C^{14} to D-xylose-1-C^{14} has been accomplished through the intermediate production of 1,2-isopropylidene-D-xyloglutaric dialdehyde (85). The unlabeled 1,2-isopropylidene dialdehyde, when used in the cyanohydrin synthesis with C^{14}-labeled cyanide, yielded 1,2-isopropylidene-D-glucuronic-6-C^{14} lactone, from which D-glucose-6-C^{14} was prepared by reduction (86). L-Ascorbic acid-1-C^{14} has been prepared from labeled D-sorbitol (87), and D-fructose-1,6-C^{14} from labeled D-mannitol (88). The 1,6-labeled fructose was converted to potassium D-arbonate-5-C^{14}, and ultimately to D-arabinose-5-C^{14} (89). The latter material was used in a second cyanohydrin synthesis to prepare D-glucose-6-C^{14}.

The use of certain metallic hydrides for the reduction of the sugar lactones has recently received attention. Thus lithium hydride has been applied to the reduction under anhydrous conditions of certain acid derivatives to

glycitols (90); when the method was used in the reduction of 1,2-isopropylidene-D-glucuronic lactone it gave a high yield of D-glucose-6-C^{14} (91). Sodium borohydride, which can be used in aqueous solution, reduces the lactones first to the sugars, and then to the alcohols (92). The method is promising, but at present does not give as high yields of the sugars as the modified sodium amalgam reduction.

Distribution of carbon-14 in sugars.—One of the most important uses of tracer studies lies in determination of the carbon-14 in the various positions of the molecule of the product. Several degradation systems, some of them involving a fermentation step, have been described (93 to 96). Probably the most convenient method for the determination of the activity of carbon 1 consists in oxidation of the sugar with oxygen in alkaline solution and analysis of the resulting aldonic acid in the form of a suitable derivative [(89), e.g., potassium arabonate from glucose, fructose, or mannose]. The activity of carbon 2 can be determined from the specific activity of the carbon dioxide obtained by degradation of the aldopentonic acid with hydrogen peroxide (96). The activities at carbons 3, 4, and 5 can be determined from the specific activities of the carbon dioxide produced by lead tetraacetate oxidation of the methyl hexopyranoside, the methyl pentopyranoside, and the pentose phenylosatriazole respectively (96, 97). The activity at carbon 6 is conveniently determined by periodate oxidation of the aldonic acid and isolation of the resulting formaldehyde by the dimedone compound (89).

Isotope effects.—Use of carbon-14 as a tracer is based on the premise that the isotopically labeled substance or atom will behave exactly like the unlabeled substance. Considerable deviation from this postulate has been found (98, 99). The effect varies from 0 to about 15 per cent when the labeled carbon is directly involved, but is negligible when the labeled atom is not involved. The effect is primarily one of reaction rate, and hence it can be neglected in all intermolecular reactions that go to completion. However, caution must be observed in drawing conclusions from tracer experiments in chemical or biological systems.

TRANSGLYCOSIDATION

Enzymic processes.—The classical synthesis of polysaccharides of the starch-glycogen type (100, 101, 102) established the fact that glucose-1-phosphate, when catalyzed by a suitable enzyme, has the ability to supply glucosyl radicals to a growing polymer chain without the intermediate production of the free sugar. The concept of this mechanism led to the enzymic synthesis of sucrose by reaction of D-glucose-1-phosphate and fructose in the presence of "sucrose phosphorylase" and ultimately to the synthesis of a variety of new oligosaccharides from D-glucose-1-phosphate (103, 104). It soon appeared that sugar phosphates are not the only substances that can act as intermediates in the enzymic synthesis of polysaccharides. An early indication of this fact is the observation by Pigman (105) that certain enzymes have a synthesizing action on maltose in forming unfermentable

saccharides, but that a similar effect is not found in glucose solutions. Later work has shown that glycosyl radicals can be transferred by suitable enzymes from one saccharide to another, with utilization of the energy of the glycosidic bond of the first saccharide to form the glycosidic bond of the new substance. Once this principle of transglycosidation had been recognized, the effect became obvious in many systems; its study has been facilitated by the new techniques of chromatography, and in some cases the mechanism of the synthesis has been clarified by use of isotopically labeled tracers.

Enzymic transglycosidation appears to have some similarity to acid-catalyzed "reversion" whereby higher saccharides are formed from the lower, but the two processes differ in that equilibrium with the sugar is established only in the latter. The term transglycosidation has also been used to designate the chemical process consisting of the conversion of one "anomer" to another, usually by the action of an acidic catalyst [12 (p. 85)]. Thus β-melibiose octaacetate may be prepared by the action of titanium tetrachloride on β-6-β-galactosyl-glucose octaacetate (106). In order to avoid confusion it may be desirable to restrict the term to enzymic processes.

Typical syntheses.—Enzymic transglycosidations, even including some that take place through the phosphates, follow the general pattern:

$$\begin{array}{l} \text{Glycosyl derivative A} + \text{enzyme-H} \rightarrow \text{glycosyl-enzyme} + \text{residue A-H} \\ \text{Glycosyl-enzyme} + \underset{\text{(acceptor)}}{\text{ROH}} \rightarrow \underset{\text{(product)}}{\text{glycosyl-OR}} + \text{enzyme-H} \end{array}$$

With the sugar phosphates, residue A is inorganic phosphate; with maltose as a source of glucosyl groups, residue A is glucose (107, 108). Sucrose can act as a source of either glucopyranosyl or fructofuranosyl groups, and leave residues of fructose or glucose respectively.

If the acceptor, ROH, is water, the product formed is a sugar:

$$\text{Glycosyl-enzyme} + H_2O \rightarrow \text{sugar} + \text{enzyme-H}$$

Thus the enzymic hydrolysis of a glycoside or saccharide can be considered a process of transglycosidation (109). If the acceptor is an alcohol, the product is a glycoside. For example, an enzyme from the green leaves of *Salix sachalinensis* or *Populus alba* in the presence of methanol converts phenyl β-D-glucoside to methyl β-D-glucoside (110). Similarly, transfructosidation by a yeast invertase in the presence of methanol and sucrose yields methyl β-D-fructofuranoside (111). If the acceptor is a saccharide, the product is a higher saccharide. Transglycosidation reactions are ordinarily in competition with the hydrolytic reaction, and in many cases synthetic oligosaccharides can be detected only when the hydrolytic reaction is incomplete (17, 18, 112). Since the transformations in an enzyme system may give rise to acceptors other than the primary acceptor, the ultimate product may be a mixture of complex saccharides. Thus, from maltose, the transglucosidase of *Aspergillus oryzae* synthesizes the disaccharide isomaltose, the trisaccharides 4-α-isomaltosyl-D-glucose (panose) and 6-α-isomaltosyl-D-glucose (dextrantriose), and the tetrasaccharide 4-α-dextrantriose-D-glucose (29). It was shown by

use of glucose-C^{14} in the reaction mixture, that glucose serves as an acceptor, but that the enzyme action involves the transfer of α-glucosyl units from 1,4-glucosidic compounds to the 6-position of the acceptor saccharides, resulting in the synthesis of 1,6-oligosaccharides. An enzyme from a strain of *Meningococcus* reacts with maltose and phosphate with the production of glucose, and β-D-glucose-1-phosphate (113). The latter compound and the enzyme have been shown to react with xylose to give a glucosido-xylose. Apparently, the concerted action of the β-D-glucose-1-phosphate, the enzyme, and the acceptor is required. The recently reported presence of O-α-D-galactopyranosyl-myo-inositol in sugar beet juice containing myo-inositol (114) may be the result of a transgalactosidation in which the inositol is the acceptor, and the raffinose the substrate. Thus:

Raffinose + enzyme-H → galactosyl-enzyme + sucrose
Galactosyl-enzyme + myo-inositol → galactosyl-inositol + enzyme-H

It can be seen from the following tabulation that many new oligosaccharides have been obtained by enzymic transglycosidation. Some of the products have not been fully identified as yet, and the structures are provisional. The field offers great opportunity for future work, especially with enzyme systems related to the bacterial polysaccharides, cellulose, pectins, gums, and uronides.

Dextran

Blood plasma expander.—In response to an urgent need for suitable blood plasma expander, an extensive research program has been conducted on the production of so-called clinical dextran. This material is obtained by partial hydrolysis and fractionation of native dextran, a polysaccharide formed from sucrose by certain bacteria (125). An excellent bibliography has been prepared on the chemical, microbiological, and clinical aspects of the use of dextran as a blood plasma expander (126). To insure a safe and effective product, the molecular weight of clinical dextran must be closely controlled. The commercial product has a number average molecular weight of 40,000 to 60,000, and a weight average molecular weight of 75,000 ± 25,000 (127). A formalized procedure using a commercially available light-scattering photometer was developed for determining weight-average molecluar weight in clinical solutions of dextran (128). The procedure, which is now on a satisfactory routine basis, should prove useful in studying other poly-disperse systems. As a result of the manufacturing process, each molecule of clinical dextran has one and only one reducing end group. This relationship makes possible the calculation of number average molecular weights from end group analyses. Methods used for the determination of end groups include reaction with radioactive cyanide (53), dinitrosalicylic acid (129), and copper reagents. The most acceptable method at present seems to be copper reduction measured by the method of Somogyi (130), standardized on the assumption that one mole of dextran has the same copper-reducing power as one mole of isomaltose (131, 132).

TABLE I

OLIGOSACCHARIDES PRODUCED BY TRANSGLYCOSIDATION

Source	Primary Substrate	Products
	Transglucosidations	
Aspergillus oryzae	maltose	Isomaltose, 6-glucosyl-maltose, 6-glucosyl-isomaltose, 6-isomaltosyl-maltose (29, 109)
A. niger	maltose	4-isomaltosyl-glucose (115)
Escherichia coli	maltose	maltotriose, maltotetrose (108)
Neisseria meningitis	maltose (β-D-glucose-1-phosphate intermediate)	In presence of xylose: glucosyl-xylose (113)
Honey invertase (glucoinvertase)	sucrose	2-maltosyl-fructose (erlose), higher saccharides (116, 117)
Leuconostoc mesenteroides	sucrose	dextran (118, 119)
Leuconostoc mesenteroides	sucrose	Glucosyl-fructose (leucrose) (120)
	Transfructosidations	
A. oryzae	sucrose	1-inulobiosyl-glucose, 1-inulotriosyl-glucose (121)
A. oryzae	sucrose	In presence of raffinose: fructosyl-raffinose, pentasaccharide (121)
Yeast invertase	sucrose	fructosyl-sucrose [kestose (16)] and other oligosaccharides (117, 122)
Artichoke tubers	inulin	In presence of sucrose: fructosyl-sucrose, higher saccharides (123)
	Transgalactosidations	
A. oryzae	lactose	galactosyl-lactose (112)
Saccharomyces fragilis	lactose	galactosyl-glucose, galactosyl-galactose, galactosyl-lactose (124)

Structure of dextrans.—Early methylation and degradation studies indicated that the dextran produced by *Leuconostoc mesenteroides* is composed of glucose units in α-1,6 linkage, with occasional branching at carbon 4. The ratio of 1,6- to non-1,6 linkage may be obtained simply from the amount of the formic acid produced by periodate oxidation (133, 134). Periodate oxidation of the dextrans from 100 strains of microorganisms, showed that 50 to 97 per cent of the linkages are of the 1,6-glucopyranosidic type (135). The

presence of a high percentage of non-1,6 linkages is indicative of a highly branched structure, but the existence of chains of non-1,6 links, possibly of the amylose type, is not excluded. By periodate oxidation of dextran, and study of the oxidation product, it has been shown that dextran contains units resistant to periodate oxidation, presumably glucose units substituted at carbon 3 (136, 137, 138).

Antigenic properties.—Samples of clinical dextran imported from Sweden and Great Britain have given reactions of an allergic type in a considerable number of individuals, but the product prepared from the dextran of *L. mesenteroides* NRRL-B512 gave very few reactions, and these were mild (139). It has been found that native and clinical dextrans having different branching ratios are antigenic in man and that certain dextrans cause production of two types of antibodies (140, 141). It may be significant that the dextran from *L. mesenteroides* NRRL-B742 was found to consist of at least two discrete fractions which differ in specific rotation, infrared absorption, and other properties (137, 142).

Metabolic fate.—In order to establish that clinical dextran is not stored in any organ of the body, it was necessary to ascertain its metabolic fate. For this purpose, C^{14}-labeled dextran was synthesized from randomly labeled sucrose-C^{14} and given intravenously to rats, dogs, and finally to human beings. The results showed that the material is metabolized and ultimately eliminated (74).

Reaction Mechanisms

Mutarotation of sugars.—Many processes of biological importance take place through intermediates common to the mutarotation reaction; hence this reaction has continued to receive attention. Rate constants for the conversion of the ring forms of sugars to the aldehyde forms, calculated from polarographic measurements, have shown that the energy of activation (16.5 ± 0.5 kcal.) for the rupture of the pyranose ring, is the same for glucose, galactose, mannose, arabinose, and xylose. The entropies of activation were found to be low and positive (143). It has long been known that the rate-determining step in the mutarotation reaction is the formation of the open-chain modification, which then rapidly recyclizes. There are several concepts as to the manner in which the open-chain modification is formed. Years ago Lowry advanced the idea that both an acid and a base catalyst are necessary, and that the open-chain modification is formed by simultaneous addition and elimination of a proton (144). Other workers have held that separate acid- and base-catalyzed mechanisms act step-wise. The step-wise mechanism through either the conjugate acid or the conjugate base form of the sugar should conform to second order kinetics, whereas a concerted mechanism involving both an acid and a base catalyst should require a third order reaction. A recent study of the mutarotation of glucose in water-methanol mixtures containing acetate buffers shows better agreement with second order, than with third order kinetics (145). However, the kinetics of the

mutarotation of tetramethyl glucose, catalyzed by phenol and pyridine in benzene solution, were found to be third order (146).

These results, obtained under widely different experimental conditions require at least three mutarotation mechanisms (Figure 1). Presumably the acid- and the base-catalyzed mechanisms function side by side, and the overall reaction can be regarded as the sum of several parallel and competing reactions, the rates of which depend on the acid and base catalysts present. Parallel reactions are possible for all cyclic and ionic species of the sugar, and hence the system is far more complex than the diagrams indicate (147).

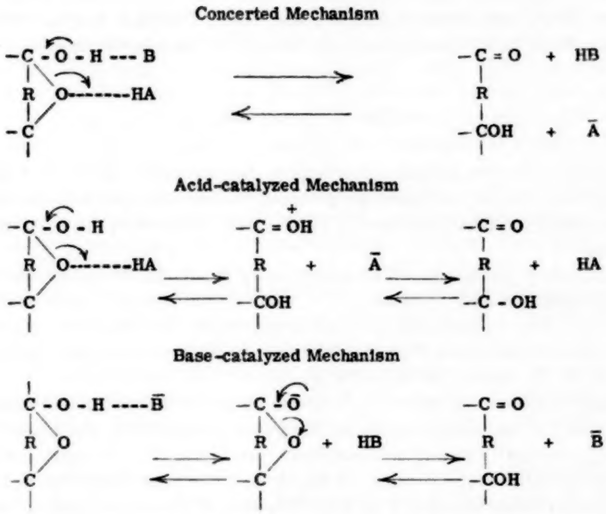


FIG. 1. Mutarotation mechanisms.

A unique proof for the existence of the concerted mechanism in the case of the mutarotation of tetramethylglucose was obtained by use of a special bifunctional catalyst (148). The catalyst, 2-hydroxypyridine, contains a nucleophilic and an electrophilic group rigidly held at the optimum distance apart for interaction with the substrate. The substance was shown to have high catalytic activity and to possess catalyst-substrate specificity approaching that of enzymes. The mechanism was presented as in Figure 2. An enzyme which accelerates the mutarotation of glucose (mutarotase) has been found in culture filtrates of *Pencillium notatum* (149). It is strongly inhibited by excess of glucose and by carbohydrates structurally similar to glucose. The action of 2-hydroxypyridine and other polyfunctional catalysts on the mutarotation of glucose (148) may prove helpful in the study of this enzyme.

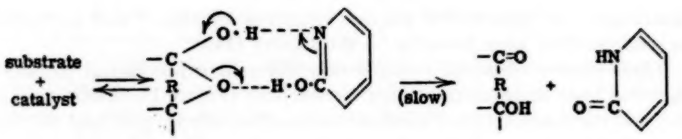
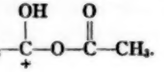


FIG. 2. Catalysis of mutarotation by 2-hydroxypyridine.

Anomerization of sugar acetates.—The anomerization of β-D-glucose pentaacetate by a mixture of acetic anhydride and sulfuric acid (150, 151), has been interpreted by a mechanism based on the bifunctional character of the anhydride, induced by the strong acid (152). The first step is the ionization of sulfuric acid in acetic anhydride to give the conjugate acid,



This reacts with the acetate according to the equation shown in Figure 3. Although other interpretations are possible, this mechanism is in accord with the demonstrated effectiveness of a bifunctional catalyst in the mutarotation reaction.

Acetolysis of glycosides.—The acetolysis of glycosides by acetic anhydride in the presence of acid catalysts (153), can be interpreted in similar manner (Figure 4). The replacement of—OR in glycosidic combination, but not in ether combination shows that the electron-releasing effect of the ring oxygen must aid in the release of the—OR group.

Anomerization of glycosides.—A mechanism similar to the acid-catalyzed mechanism for the mutarotation of the sugar accounts for the interconversion of glycosides by strong acid catalysts (see Figure 5). The aglycone group R can be another sugar residue, in which case the rearrangement gives rise to a different disaccharide [12 (p. 85)]. The role of the ring oxygen in accepting the electrons from the glycosidic carbon explains why glycosides rearrange, whereas sugar ethers do not.

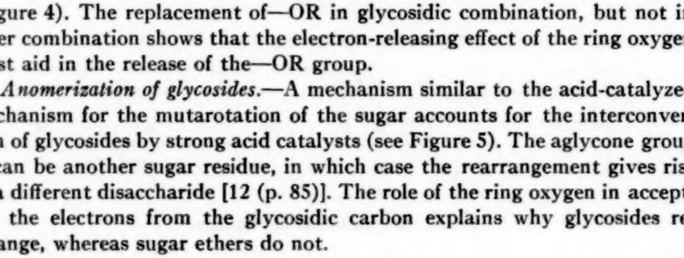


FIG. 3. Anomerization of sugar acetates.

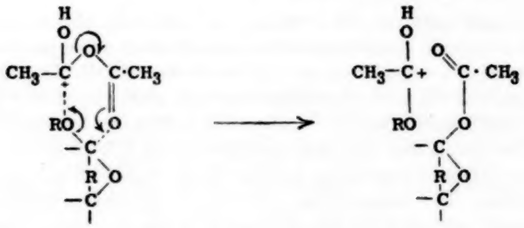


FIG. 4. Acetolysis of glycosides.

Mutarotation of nitrogen glycosides.—The mutarotation of nitrogen glycosides and glycosylamines, unlike the sugars, is not appreciably catalyzed by bases, but is strongly catalyzed by acids (147, 154). This fact may be ascribed to the difference in the tendency of amino nitrogen and hydroxyl oxygen to accept and release protons. The mutarotation reaction of nitrogen glycosides and glycosylamines involves the formation of a highly reactive imonium cation $-\overset{|}{C}=\overset{+}{N}RR'$. Depending upon the conditions and the substances present, this cation may react with materials from the environment or undergo rearrangement by intramolecular changes. Thus reaction with water leads to hydrolysis; reaction with a second molecule of glycosylamine produces the diglycosylamine (147, 155). Reaction of the cation with a hydroxyl group from within the molecule gives the cyclic forms of the amine and completes the mutarotation; an electronic shift in the cation, with migration of the proton from the adjacent carbon, gives an enolic derivative analogous to the enediol of the sugar (Amadori rearrangement). Apparently other intramolecular rearrangements can occur in acetic acid (156) or in pyridine (157).

In a study of the interconversion of a number of N-arylglycosylamines, it was shown that one substance, believed to be a furanoside, is formed in anhydrous ethanol, and that another, believed to be a pyranoside, is formed in aqueous ethanol (158). The work illustrates the fact that the ring in N-glycosides has greater lability than that in O-glycosides. The effect of moisture in directing the product to a different ring form than that obtained from anhydrous solvents appears somewhat similar to the directing effect of calcium chloride in the crystallization of D-mannofuranose calcium chloride (159).

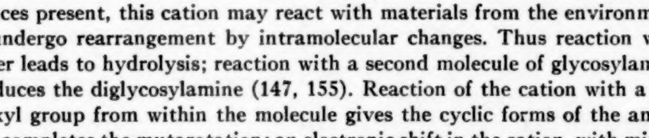


FIG. 5. Anomerization of glycosides by strong acids.

Reaction of sugar acetates with secondary amines.—Neighboring groups and successive electronic displacements frequently play an important part in the reactions of the sugars and the glycosylamines (160). Treatment of pentaacetyl-D-glucose (I) with piperidine has been shown to give N-(3,4,6-triacetyl D-glucosyl)-piperidine (IV), a substance with a free hydroxyl at carbon 2 (161). The compound was also produced from 2,3,4,6-tetraacetyl-β-D-glucopyranose (II) and piperidine, but not from N-(2,3,4,6-tetraacetyl-D-glucosyl)-piperidine. The mechanism, involving an orthoester intermediate (III), satisfactorily accounts for the formation of the compound (see Figure 6.)

The assumption of an orthoester intermediate seems warranted in view of the prior observation (162) that methyl orthoacetates of the sugars (V), on successive treatment with hydrochloric acid and silver hydroxide give products with a free hydroxyl at carbon 2 (VI) (Figure 6):

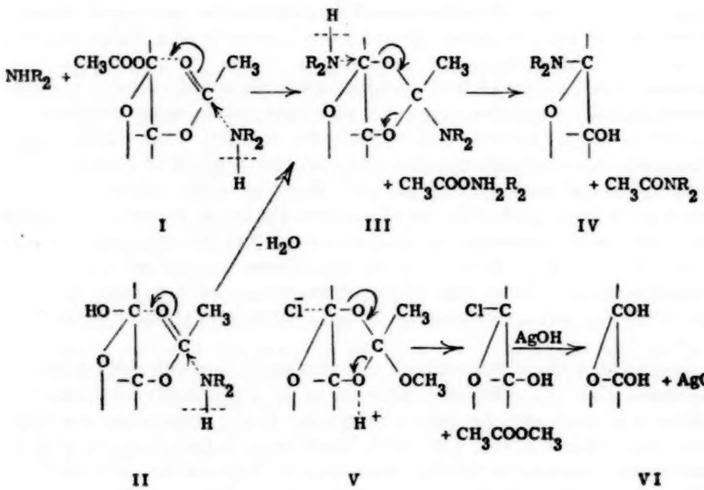


FIG. 6. Some reactions of sugar acetates.

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CHEMISTRY OF THE LIPIDS[1]

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This review is selective and not comprehensive. No attempt is made to cover all the advances in lipid chemistry during the past year. Instead, it is desired to comment more fully upon a few only of the topics which have been prominent in published communications during this period and the years which immediately preceded it.

The first volume of a treatise on *The Lipids: Their Chemistry and Biochemistry* by Deuel (1) deals comprehensively with the chemical aspects of the different kinds of lipids and presents to biochemists and physiologists modern knowledge of lipid chemistry fully and competently.

Glyceride Composition of Natural Fats

The mode of distribution of fatty acids in the triglycerides of natural fats was discussed three years ago in this Review by Deuel (2), who gave a balanced and critical survey of the views which had been put forward on this matter up to that time. It is nevertheless desirable to return to the subject, which is of great interest in lipid chemistry, since some fresh observations have been made in the interval, and since there appears to be a fundamental difference in approach to the question between several workers in the field and the writer of this Review, who originally introduced the concept of "even distribution" of fatty acids in natural triglyceride molecules. This was in 1929, when attention was drawn to "the pronounced tendency to even distribution of the fatty acids throughout the glycerides of seed fats" (3). This tendency has been observed since to a marked degree in many natural fats, and it has been the object of the writer and his co-workers to classify the general pattern of glyceride structure according to the observed findings but not to attempt to find any numerical formula which will fit all instances. Such a formula, by nature of the case, appears most unlikely to exist, and it has been preferred (4, 5) to define the pattern of glyceride structure in more general terms. (Deuel (2) has given a summary of these which, therefore, need not be repeated here.) The only attempt (6) of the Hilditch school to predict glyceride structure from the fatty acid composition of fats consisted of certain purely empirical "computations" whereby in many cases the approximate glyceride composition of a fat can be roughly ascertained from the fatty acids present. This was intended merely to indicate how, in some instances, the lengthy experimental determination of the glycerides present might be escaped.

Other workers have implied that "even distribution" must be taken in its

[1] The survey of the literature pertaining to this review was concluded in October, 1952.

most literal interpretation (e.g., that until a given acid forms over two-thirds of the total acids in a fat, no simple glycerides with three radicals of that acid can be present; or, that when it forms 66.7 per cent of the total acids all the glycerides must contain two radicals of the acid; and so on). Thus, Dutton, Lancaster & Brekke (7) make the following criticism:

> Hilditch would have the rule of even distribution applied as a generalisation which covers "the general trend of the observed facts more or less accurately." In defining the rule, the limits are so broadly drawn as to make evaluation of its validity impossible in many instances. . . .

They add that, in their paper on the structure of soybean glycerides (7), "even distribution will be defined in strict stoichiometric algebraic terms." It can only be repeated that such strict stoichiometric algebraic interpretation has little relation to the facts of natural glyceride structure, except that it represents a limiting state towards which the glycerides of seed fats and many other fats approach, often very closely, but rarely with complete exactitude.

Doerschuk & Daubert (8) in their exhaustive separation of corn oil glycerides were able to arrive at a numerical formula which closely simulated their experimental findings and which they describe as based on "partial random distribution." It would be interesting to ascertain how far this method of computation may accord with the experimental results which have been published for many other fatty oils. Meanwhile a somewhat different approach to the problem of seed glyceride structure has been made by Barker & Hilditch (9) and Hilditch & Seavell (10). These workers submitted a number of seed fatty oils rich in linoleic or linolenic acid, or in both, to segregation by low-temperature crystallisation, and plotted the observed contents of mono-, di-, and tri-linoleo (or -linoleno) glycerides against the percentages of the respective acids in the total acids of each oil. Smooth curves for glycerides containing one or two groups of an acid (e.g. linoleic) resulted, with maxima at the points where the percentage of the acid in the total acids was respectively 33 per cent and 67 per cent. These maxima corresponded with contents of 85 to 90 per cent of the total glycerides in the oil; this figure is perhaps the best quantitative illustration yet available of the extent to which "even distribution" preponderates in the constitution of natural glycerides. The curves show that glycerides with one group of a given acid persist in small amounts after such acid forms more than two-thirds of the total acids, that those with two groups of the same acid appear in small proportions somewhat before the acid forms one-third of the total acids, and that simple triglycerides with three groups of the same acid begin to appear somewhat before it forms two-thirds of the total acids. The curves for trilinolein or trilinolenin, moreover, are identical with those relating trisaturated glycerides to content of saturated acids determined by an entirely different technique.

It has been found that mono- and di-acid glycerides of the major component acids of other seed oils are present in such proportions that they con-

form to the same graphs, for instance, in tobacco seed oil (11), groundnut oil (12), and the elaeostearic glycerides of tung oil (13). It also appears that the total contents of mono- and di-linoleo and -oleo glycerides observed by Doerschuk & Daubert (8) in corn oil conform approximately with these graphs.

Substantial accordance has in fact been reached as regards the experimental findings on the constituent glycerides of most fats which have been examined (within the limits of accuracy of the techniques yet available in this rather difficult field). It would seem desirable in future to direct our thought to the implication of these findings in relation to the biosynthesis of natural glycerides rather than to much further discussion of what appears to no small extent to be verbal misunderstanding, some part of responsibility for which may be due to the writer of this report.

A phenomenon which requires consideration along with the general distribution of fatty acids in glycerides is an apparent specificity in their configuration: when a glyceride contains two fatty acids, that which is present in lesser proportion in the total acids of the fat seems almost exclusively to be attached to the central or β-hydroxyl group of the glycerol molecule, and conversely. Thus Meara (14) and Lutton (15) observed that in Kokum butter (*Garcinia indica* seed fat) the oleodistearin is the symmetrical form, and similarly (16, 17, 18) that β-oleodipalmitin is the only isomer present in large amounts in the glycerides of the fruit coat fats of piquia and stillingia. Meara (14, 19, 20) has shown also that only the symmetrical β-oleodistearin is present in the seed fats of three species of *Allanblackia*, of *Pentadesma butyracea*, and *Palaquium oblongifolium* and that only β-palmitodistearin is obtained when the palmito-oleostearins, palmito-oleo-linoleins, or palmito-dioleins of pig body fat, cottonseed oil, or cacao butter are hydrogenated. On the other hand, Meara (16) found in piquia fruit coat fat that, in contrast to the unique presence of β-oleodipalmitin, the palmitodioleins in this fat are a mixture of the symmetrical and unsymmetrical isomers, probably in approximately equal proportions. Again, in palm oil (21) the oleodipalmitins consist of roughly equal mounts of α- and β-oleodipalmitin, whilst the unsymmetrical isomer forms over 90 per cent of the palmitodioleins of the fat. In seed fats, then, an acid present in less proportion than another uniformly takes the central or β-position in the triglyceride molecule, but important divergencies from this have been observed in two fruit coat fats (piquia and palm oil) but not in another, namely, stillingia tallow. This matter is clearly closely related to the mechanism of glyceride biosynthesis and merits further exploration.

A new method of determining mixed saturated-unsaturated glycerides has been proposed by Kartha (22). It depends on analysis of the mixture of azelao-glycerides produced when a fat is oxidised by anhydrous potassium permanganate in an organic solvent. That this had formerly (23) proved impracticable is attributed by Kartha to partial hydrolysis of the azelao-glycerides when acetone alone is used as solvent, and he finds that use of sufficient acetic acid with the acetone solution to maintain slight acidity

throughout the oxidation suppresses hydrolysis and enables the azelaoglycerides to be recovered and their proportions determined. It has meantime been proved by Begemann, Keppler & Boekenoogen (24) that when acetic acid is used in place of acetone as solvent during oxidation of unsaturated fatty esters the amount of secondary oxidation of, e.g., azelaic esters to suberic esters is much reduced. The use of acetone with acetic acid as recommended by Kartha has enabled him to recover all the monoazaleo-glycerides produced (in admixture with some diazaleo-glycerides) and thereby to determine the amount of monoazaleo-glycerides which in turn gives the molar percentage of monounsaturated glycerides in the fat. The amount of trisaturated glycerides also having been determined, the proportions of di- and tri-unsaturated glycerides can be calculated, knowing the fatty acid composition of the whole fat. This method requires the study of considerably smaller amounts of fat than are needed for partial separation by low-temperature crystallisation, and it invites further examination and confirmation by others interested in this field. The results given by Kartha for a number of fats appear to accord fairly well with those obtained by other workers by the more recent crystallisation studies, but of course they only serve to classify the glycerides in regard to the total number of saturated and unsaturated groups present in the triglycerides of the fat. Kartha, perhaps unfortunately, seems mainly concerned to employ his data to contest the rigid "stoichiometrically algebraic" concept of "even distribution" which, with others, he erroneously ascribes to Hilditch, and proceeds to evolve a computation of his own which is claimed at great length to apply to all natural fats. His argument, admittedly difficult to follow, is nevertheless open to the general criticism of over-simplification and at the same time of over-elaboration in comparison with its relatively slender basis of experimental observations.

Biosynthesis of Unsaturated Acids in Seed Fats

The saturated long-chain acids of natural fats can be envisaged as built up primarily by aldol condensation from C_2 units followed by bio-hydrogenation and bio-oxidation processes. To account on this basis for the natural production of oleic, linoleic, and other unsaturated acids (which incidentally are far more abundant in seed fats than any of the saturated acids) it was formerly supposed that they were produced by progressive desaturation of stearic acid. Leathes & Raper (25) pointed out many years ago the unlikelihood that a saturated acid was an intermediate stage in the synthesis of a much less stable unsaturated acid. This has become more apparent since in recent years it has been demonstrated that in many seeds the fats produced are the more unsaturated, the cooler the climate in which the seeds are matured: for, on the old hypothesis, desaturation or dehydrogenation must proceed to a greater extent in seeds grown at lower than at higher temperatures. The influence of environment on the unsaturation of seed fats has been amply demonstrated in the cases of linseed (26, 27) soybeans (28, 29), cottonseed (30), and sunflower seeds (31, 32). The seed fat of any given spe-

cies elaborates its own specific mixture of fatty acids [e.g., linolenic, linoleic, oleic in linseed oil, or linoleic and oleic in sunflower seed oil, with subordinate proportions of saturated (palmitic and stearic) acids in each], but the relative proportions of the specific unsaturated acids present may vary widely with the rate of development of the ripening seed. In such cases, however, great may be the variation in unsaturated fatty acid composition (and this may range, for instance, from contents of 30 to 70 per cent of linoleic acid in sunflower seed oils or of 35 to 60 per cent of linolenic acid in linseed oils); there is extremely little variation in the proportion of saturated acids in the seed fats; indeed the more unsaturated seed fats usually contain very slightly greater proportions of saturated acids than those with lower contents of unsaturated acids.

This suggests that the mode of unsaturated fatty acid formation is quite distinct from that of saturated acids and that, contrary to the older suppositions, the more unsaturated acids (e.g., linolenic or linoleic) are precursors of oleic acid. Such an explanation (33) is in harmony with the production in a given species of more unsaturated fatty acids in greater proportions under cool climatic conditions, but it should be noted that it only applies to the unsaturated members: there is no indication that stearic glycerides are produced by bio-hydrogenation of oleic glycerides. The suggestion of essentially different mechanisms of synthesis of unsaturated and saturated acids also accords with the circumstance that the natural unsaturated acids of seed fats of a given species are in general confined to those of the same carbon content (usually C_{18}), whereas their saturated acids are almost invariably a mixture of homologues with one acid (e.g., palmitic in cottonseed oil and in many other seed fats, lauric in Palmae seed fats, etc.) present in much greater proportions than the adjacent even-numbered homologues which accompany it.

It will also be recalled that Bloch (34), from *in vitro* studies with acetate and pyruvate labelled with deuterium, carbon-13 or carbon-14, has concluded that separate processes are involved in the biosynthesis of saturated and unsaturated acids.

Some Animal Milk and Depot Fats

Fats of Ruminants.—The origin of the short-chain saturated acids in ruminant milk fats and, more generally, the extent to which synthesis of milk fat may take place in the mammary gland itself are subjects which have received much attention in the past few years. The production in the gland of short-chain milk fat acids from carbohydrate was first explicitly suggested in 1941 by Reineke, Stonecipher & Turner (35), whilst a few years later Bloch & Rittenberg (36, 37) established by tracer experiments that the effective units from which synthesis of fatty acids or cholesterol proceeds are acetate units or a more reactive derivative of acetate. Much recent work by Popjak, Folley and their colleagues has proved conclusively that a great part, at all events, of the saturated short-chain acids of cow and goat milk fats is synthesised in the mammary gland from acetate or from carbohy-

drate which is first converted to acetate or pyruvate units. Comprehensive reviews of these researches have been given by Popjak (38, 39) and Folley (40, 41), and it is only possible to mention here a few of the more important results in their recent communications. The work on rabbits of Popjak & Beeckmans (42) provided more conclusive evidence than was previously available that fatty acid synthesis from small molecules proceeds in the mammary gland. In 1951 Popjak *et al.* (43) injected goats intravenously with radioactive (carboxy-C^{14})-acetate and demonstrated that radioactive saturated fatty acids up to palmitic were present in the goat milk fat; the oleic and stearic acids in the latter, however, possessed negligible radioactivity. Radioactive butyric, hexanoic, and octanoic acids isolated from the goat milk fat were oxidatively degraded to lower acids by the method of Hunter & Popjak (44), and it was thus found that the radioactivity was confined to the alternate carbon atoms 1 (carboxyl), 3, 5, and 7 of the synthesised acids, thus proving that synthesis occurred by attachment of an "acetate" unit to the carboxyl end of a preformed fatty acid. In similar experiments with lactating rabbits French & Popjak (45) compared acetate and glucose as sources of carbon for the milk fat synthesis. They estimated that in six hours about 30 to 70 per cent of the short chain acids (up to about C_{12}) of the milk fat were synthesised from acetate and that in similar conditions at least 25 per cent of short-chain acids were derived from carbohydrate. The recent results extend the earlier studies of Folley & French (46) in which mammary gland slices are employed; full bibliographical references to the numerous publications of work carried out by Folley, Popjak and their co-workers are given in the reviews (39, 41) to which reference has already been made.

The demonstration of the large extent to which synthesis of short-chain milk fat glycerides takes place in the lactating gland of ruminants must be considered concurrently with observations (47, 48, 49) that during passage of blood through the gland the blood glycerides disappear in quantities commensurate with the milk fat formed, and with the known facts of the specific chemical constitution of milk fat glycerides in general. Hilditch (50, 51, 52) has maintained the view that the characteristic glyceride structure of milk fats indicates that they are produced by indiscriminate or random conversion in the lactating gland of the oleo groups in preformed (blood) glycerides into the shorter-chain acyl groups. He has suggested also that oleic acid residues, in glyceride combination, are broken down in the udder by oxidation and reduction processes giving rise to the traces of $\Delta^{9:10}$ unsaturated acids of the C_{10}—C_{16} series present in ruminant milk fats and also to the short-chain acids. The latter part of this conclusion does not harmonize with the facts which emerge from Folley & Popjak's recent studies, yet milk fat glyceride structure involves the presence of short-chain groups where oleo-groups would occur in analogous (blood or depot) fatty glycerides of the animal. Hilditch (53) has, however, pointed out that "conversion" of an oleo-group into a short chain acyl group might take place either by the chemical "shortening" of an oleic group previously postulated or by the replacement of an oleic group by a short-chain acid synthesised in the gland

by the operation of acyl interchange. The interchange of acyl groups in mixed glycerides is known to proceed readily under various conditions *in vitro* and conceivably may equally proceed in active tissues by enzyme catalysis. This suggestion appears to take account both of short-chain acid synthesis in the mammary gland, of the specific glyceride structure of ruminant milk fats, and of the production of milk fat from blood glycerides which, on this view, serve as it were to absorb the synthesised short-chain acids into preformed glycerides during passage of the latter through the udder. The simultaneous presence of minor amounts of Δ^9-decenoic and -dodecenoic glycerides in ruminant milk fats nevertheless suggests that, to a lesser extent, chemical shortening of oleo-glyceride groups probably also takes place. Failure up to the present to detect radioactive oleic groups in milk fats produced by use of labelled acetate or carbohydrate may also be an indication that the oleic acid of milk fats (which is incidentally the preponderating acid in these fats) enters the lactating gland entirely as performed oleo-glycerides.

A comprehensive statistical survey of seasonal variations in the fatty acid composition of New Zealand butterfats has been made by Hansen & Shorland (54), who consider that the results of their study would serve as a useful guide for predicting the fatty acid composition of New Zealand butterfat for any given season of the year. The authors are able to associate the plane of nutrition with variations in the component acids of the fat: at high dietary levels a lowered content of C_{18} unsaturated acids is accompanied by increased content of the lower saturated acids, whilst when food is more scarce the content of C_{18} unsaturated acids rises. This view seems to resolve the seeming difference between the composition of New Zealand and English butter fats in the respective early summer seasons: in New Zealand cows are pasture-grazed all the year round and their food, therefore, becomes more abundant in early summer (September to January) with corresponding reduction in the C_{18} unsaturated acid content of the milk fat, whereas the stall-fed English cattle encounter a lower plane of nutrition on going to spring pasture and a sudden increase in the C_{18} unsaturated acid content of the milk takes place.

Occurrence of branched-chain acids in ruminant fats.—Hansen & Shoreland (55, 56) have recently observed that small proportions of branched-chain saturated acids are present in butterfats, including two (methylhexadecanoic) acids of the formula $C_{17}H_{34}O_2$, a trimethyl- or tetramethyl-substituted C_{20} (eicosanoic) acid, and a methylheptadecanoic acid, $C_{18}H_{36}O_2$ (57). Hansen, Shorland & Cooke (58) subsequently isolated from beef fat small amounts of a C_{17} methyl-substituted acid identical with one of those isolated from butterfat and in their latest communication (59) have reported the presence in external tissue fat of sheep of the same acid, which in this instance has been shown to be α-14-methylhexadecanoic acid identical with that isolated from wool grease by Weitkamp (60). The discovery of these very minor components in butterfat and in the reserve fats of oxen and sheep is noteworthy, even though they are of the nature of trace components. Precise determination of their proportions is difficult, but Hansen, Shorland & Cooke estimate that α-14-methylhexadecanoic acid constitutes not less than

0.2 per cent of the total fatty acids in the sheep fat. The apparent resemblance of these branched-chain acids to some of those which have been previously isolated from wool wax and from tubercle waxes may be thought to suggest that their presence in traces in the milk fat and depot fats studied by Shorland and his colleagues may be similarly due to small proportions of animal waxes which find their way into the glycerides of the respective tissues. However this may be, the isolation of such minute quantities of specific individuals acids is an admirable indication of the resources in technique which are now at the disposal of investigators of natural lipids.

Occurrence of trans-isomers of oleic and other unsaturated acids in animal fats.—Up to the present time it has seemed that natural unsaturated fatty acids were produced exclusively in the *cis*-configuration, the only exception being vaccenic acid present in traces in certain ruminant animal fats. In 1944 Millican & Brown (61) scrutinised the individuality of the oleic (*cis*-octadec-9-enoic) acid present in a number of natural fats after careful isolation and purification by a combination of fractional distillation and low-temperature crystallisation of methyl esters. Whilst they found only oleic acid in a number of vegetable fatty oils they observed evidence of other isomeric acids in the octadecenoic acids of beef tallow, lard. adrenal phosphatides, pig liver lipids, and human depot fat. Swern, Knight & Eddy (62), applying the infrared spectrophotometric examination of fatty acids recently described by Swern *et al.* (63) to ox pleural and kidney fats and to the commercial oleo oil and oleo stearin separated from beef fats, have now found that the content of *trans*-acids (mainly elaidic with some vaccenic) is considerably greater than was hitherto supposed. They estimate that some 10 to 15 per cent of the mono-unsaturated acids in beef depot fat consists of *trans*-isomerides. Although careful to point out that any explanation offered must be considered highly tentative, they put forward the suggestion that *cis-trans* isomerisation may be the consequence of oxidative action on *cis*-unsaturated constituents of the fat, with or without double-bond shift, by unsaturated fatty acid oxidases known to be present in animal tissues (64). This view is attractive, in the writer's opinion, not only because it correlates the occurrence of elaidic acid in these fats with the concurrent presence of other structural isomers ("vaccenic acid") of oleic acid in which shifting of the double bond has taken place (65, 66); but also because some such quasi-chemical alteration of the natural *cis*-acid seems necessary to account for the presence of *trans*-isomerides in a natural fat.

Lipids of the horse.—The horse stands somewhat apart from other domestic animals, and indeed from most other herbivorous mammals for whom data is yet available, in that it readily stores in its fats linolenic as well as linoleic and oleic acids derived from its food. This has become evident from work carried out in the past few years in New Zealand, England, and Sweden and, although some of the results were made known before 1951, it seems useful to review here the general picture which has now been presented. Most of the studies were on fats from horses which had been wholly fed at pasture, the grass-fats of which contain considerable proportions of linolenic acids.

Broadly speaking the component acids of these fats show great similarity: linolenic acid forms 16 to 17 per cent of the total acids whilst the linoleic content is much smaller (5 to 8 per cent); oleic (33 to 36 per cent), palmitic (25 to 27 per cent), and stearic (4 to 7 per cent) are of the order common in many "non-stearic-rich" animal fats, but hexadecenoic acid (7 to 11 per cent) is distinctly higher than in the depot fats of most land animals. This statement holds for the following horse fats from pasture fed animals: English milk (Hilditch & Jasperson, 67), New Zealand intestinal and bone (Brooker & Shorland, 68), and English mesenteric (Gupta & Hilditch, 69). Holmberg & Rosenqvist (70), however, observed that the kidney and bone fats from a Swedish horse fed in the last months before killing on hay and oats, only contained 2 to 4 per cent of linolenic with 15 to 22 per cent of linoleic acid, the general composition, however, being otherwise similar to the above. Subsequently Holmberg & Rosenqvist (71) have studied the mesenteric fat of an Icelandic pasture-fed horse and find that its component acids are closely similar to those recorded for other pasture-fed horses (linolenic 17, linoleic 5, oleic 38, palmitic 27, stearic 4, and hexadecenoic 4 per cent).

This interesting series of figures shows a rather remarkable constancy of linolenic acid in horse fat glycerides so long as there is an adequate supply of linolenic glycerides in the diet (pasture grasses). Gupta & Hilditch (69) determined the component glycerides of horse mesenteric fat and found that their structure approximated to the general widely distributed type characteristic of most natural reserve fats, and differed correspondingly from that of "stearic-rich" depot fats of oxen, sheep, and some other mammals.

In contrast to the depot fats, horse liver lipids have been found by Bruce & Shorland (72) to resemble in their general fatty acid composition those of the pig, ox, or sheep (73): the liver glycerides more or less resemble the mesenteric or other body glycerides, but the liver phospholipids contain less hexadecenoic acid than the glycerides but more stearic and highly unsaturated C_{20} and C_{22} acids. On the other hand, in the horse, linolenic acid is present in much greater proportions in the liver glycerides than in the liver phospholipids, whereas the reverse holds for the content of linoleic (octadecadienoic) acid in these two fats.

Some animal bone fats.—In addition to their study of porous bone and gelatinous marrow fats of the horse (70), Holmberg & Rosenqvist have now presented similar data for the component acids of similar fats from sheep and pigs (74). They conclude that, although similar in a certain degree to other fats (depot or liver glycerides) of the same animal, bone fats form a distinct group characterised notably by the circumstance that, in contrast to these other fats, the contents of palmitic acid or of the total C_{16} acids (palmitic+hexadecenoic) are not independent of the mean unsaturation of the C_{18} acids. They also contain significant, although very minor, proportions of lauric and possibly capric and caprylic acids.

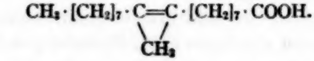
Assimilation of whale oil by pigs.—Garton, Hilditch & Meara (75) studied the depot fats of a pig whose diet had included large quantities of whale oil and found that these consisted of a mixture of whale oil with the

typical fat synthesised by the pig from nonfatty food. Simple crystallisation of the perinephric fat from acetone at −40°C., for example, gave 26 per cent of a soluble fraction close to whale oil in composition with 74 per cent of deposited glycerides closely similar in composition to those produced in the pig by synthesis from a diet low in fat.

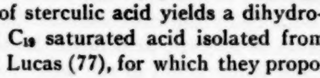
Miscellaneous Fats and Fatty Acids

Some unusual natural fatty acids.—In recent issues of these Reviews attention has been drawn to a number of interesting and unusual fatty acids which have been isolated from vegetable or animal sources. Fresh instances of this kind continue to be reported at frequent intervals, and it is fascinating to find how, here and there, fatty acids are elaborated in a particular natural species which are quite different from those which overwhelmingly preponderate in nature. This applies perhaps especially to natural unsaturated acids, of which oleic and linoleic acids are of course the most common and widespread, accounting together for much the greatest quantity of all the unsaturated fatty acids produced in natural fats. The score or so of other natural unsaturated acids which have also been observed, and to which newcomers are steadily being added, are thus collectively but a very small proportion of the whole; except for a few such as linolenic, elaeostearic, erucic, and petroselinic acids, any one of these acids forms a minute proportion of the total annual natural production of fatty compounds, and its occurrence is frequently limited to a single botanical species. It is notable that most frequently, although by no means invariably, such acids contain one or other of the two halves of the oleic acid structure, that is to say, they include either the terminal grouping $CH_3 \cdot [CH_2]_7 \cdot CH=$ or the terminal grouping $=CH \cdot [CH_2]_7 \cdot COOH$.

A most unusual acid of this kind is that present in the seed fat of *Sterculia foetida* with the composition $C_{19}H_{34}O_2$. The structure of this acid has hitherto been uncertain but Nunn (76), who suggests for it the name "sterculic acid," has now established that it is ω-(2-*n*-octyl*cyclo*prop-1-enyl)-octanoic acid.



Here, therefore, we have an analogue of oleic acid in which the central double bond is concurrently part of a cyclopropene ring system. Mild hydrogenation of sterculic acid yields a dihydro-acid which is apparently the same as the C_{19} saturated acid isolated from *Lactobacillus arabinosus* by Hoffmann & Lucas (77), for which they proposed a cyclopropane structure.

 where $x + y = 14$.

Ximenynic acid, $C_{18}H_{30}O_2$, isolated from the seed oils of three South African species of *Ximenia* by Ligthelm & Schwartz (78) and stated by these

authors to contain a double and a triple bond, has now been shown by Ligthelm, Schwartz & von Holdt (79) to be octadec-11-en-9-ynoic acid, $CH_3 \cdot [CH_2]_5 \cdot CH{=}CH \cdot C{\equiv}C \cdot [CH_2]_{17} \cdot COOH$. When heated with alkali at 180°C. the acid isomerises to a conjugated trienoic acid.

Another interesting natural fatty acid is the hydroxymonoethenoid C_{18} acid present to the extent of about 6 per cent in the seed oil of *Strophanthus sarmentosus*, which Gunstone (80) has shown to be 9-hydroxy-octadec-12-enoic acid. It is thus isomeric with ricinoleic (12-hydroxy-octadec-9-enoic) acid of castor oil, but the ethenoid and hydroxyl groups are, as it were, in reversed positions in the two acids.

Octadeca-6,9,12-trienoic acid, which bears the same structural relationship to petroselinic acid that ordinary linolenic acid has to oleic acid, was found many years ago by Eibner, Widenmayer & Schild (81) to occur in the seed fat of the evening primrose, *Oenothera biennis*, but has not been observed elsewhere. Riley (82) has confirmed the constitution of this acid and reported that it forms 10 per cent of the total component acids of the fat, the remainder being ordinary linoleic (octadeca-9,12-dienoic) acid (the chief component, 72 per cent of the whole) with oleic (7 per cent) and saturated (11 per cent) acids.

Riley has also contributed to our knowledge of the conjugated tetraethenoid parinaric (octadeca-9,11,13,15-tetraenoic) acid which is present in the seed fat of *Parinarium laurinum*. The acid forms about 54 per cent of the total acids of the seed fat (83), elaeostearic (30 per cent) being another major component, the rest including small amounts of oleic, palmitic, octadecadienoic, and stearic acids. The component acids are less evenly distributed in the glycerides of the fat than is usual in seed fats (84). Triparinarin forms over 20 per cent of the glycerides but elaeostearodiparinarin (nearly 30 per cent) is the main component, with about 15 per cent each of oleo- and saturated -elaeostearoparinarins. When methyl parinarate is hydrogenated, the primary reaction consists of simultaneous addition of either two or three molecules of hydrogen, yielding nonconjugated octadienoates (containing the unsaturated system $—CH{=}CH \cdot CH_2 \cdot CH_2 \cdot CH{=}CH—$) and octadecenoates respectively (85). Only comparatively small amounts of conjugated octadeca-dienoates and trienoates are formed, and very little methyl stearate appears until all the polyene esters have been converted into octadecenoates. The hydrogenation of the conjugated tetraethenoid parinarate system is thus strictly analogous to that of elaeosterates, in which the initial stage is addition of two molecules of hydrogen to one of the conjugated triene elaeostearate with production of considerable quantities of octadeca-11-enoate (86, 87).

In the component glycerides of tung oil (from *Aleurites fordii* and *A. montana*), the acids of which contained in different instances from 72 to 82 per cent of elaeostearic acid, elaeostearic groups were present in all the triglycerides present, the bulk of the oil consisting of oleo-, linoleo-, or saturated dielaeostearins with from 25 to 50 per cent of trielaeostearin (13).

Deca-2,4-dienoic acid, which contributes about 8 moles to every 100

moles of stillingia oil, the seed fat of *Sapium sebiferum*, was found by Crossley & Hilditch (88) to be present therein in the form of mixed triglycerides in which one deca-2,4-dienoic group is associated with two linoleic or linolenic groups. It thus behaves as if it were one of the long-chain acids so far as its mode of assembly into mixed glycerides is concerned. The acid itself when subjected to the action of alkali at 180°C., was observed by these workers (89) to undergo shift of the conjugated diene system, which tends under these conditions to migrate (mainly without separation of the conjugated double bonds) away from the carboxyl group to which it was originally contiguous.

Eicosa-11-enoic acid, which has been earlier reported to accompany erucic acid in much smaller proportions in rape seed and some other oils, has now been observed to exceed erucic acid in amount in certain *Brassica* fats. Youngs *et al.* (90) found that in the oil from Argentine rape seed grown in Western Canada eicosenoic formed 11 per cent, and erucic acid only 7 to 10 per cent, of the fatty acids. Hoffmann, Zuckerman & Grace (91) found that in the oil from weed screenings from Canadian cereal crops the acids present (in addition to oleic, linoleic, linolenic, and saturated) included 13 per cent of eicosenoic and 8 per cent of erucic; since charlock was the main constituent (53 to 77 per cent) of the weed seeds, the amount of erucic acid in the charlock seed fatty acids cannot have been more than 17 per cent.

The triene C_{16} acid present in the fatty oil in rape leaf (92) has now been shown to be hexadeca-7,10,13-trienoic acid by Heyes & Shorland (93).

It is appropriate to mention here the important contribution by Herb, Witnauer & Riemenschneider (94) on the isolation of eicosapentanoic and docosapentaenoic acids from adrenal lipids or other natural sources by combined adsorption and distillation techniques; spectrophotometric data for the pure acids after alkali isomerisation are given. The methods adopted for alkali isomerisation and subsequent spectrophotometric examination of linoleic, linolenic and arachidonic acids have been critically reviewed by Brice *et al.* (95), and a careful restandardisation of the extinction coefficients of the respective pure acids (isolated entirely by physical methods) has been made: certain minor alterations are recommended in the techniques hitherto used and certain precautions (notably corrections necessary to allow for extraneous background absorption at the wave lengths employed) are indicated.

To conclude this section reference may be made to two recent reports of the occurrence of natural fatty acids of unusually high molecular weight. Bergmann & Swift (96) have observed that the lipids of two species of sponges are characterised by containing, in addition to the usual saturated monoethenoid and polyethenoid acids with from C_{16} to C_{22} carbon atoms, substantial proportions (30 to 50 per cent of the total acids) of polyethenoid C_{26} and C_{28} acids. The constitution of the complex mycolic acids (containing about 90 carbon atoms in the molecule) from the wax of *Mycobacterium tuberculosis*, which were first isolated by Anderson and colleagues (97), has been partially unravelled by Lederer and co-workers (98, 99, 100). Three of these acids have now been isolated, all of which are derived from *n*-hexacosanoic

acid by substitution at the carbon atom next to the carboxyl group:

$$C_{24}H_{49} \cdot CH(CHOH \cdot R)COOH.$$

The alkyl group R is represented in one acid as $C_{60}H_{121}(\pm 5CH_2)$, whilst the other two mycolic acids are methoxyl and hydroxyl derivatives with R respectively $C_{60}H_{120}(OCH_3) \pm 5CH_2$ and $C_{60}H_{120}(OH) \pm 5CH_2$.

Syntheses of some unsaturated fatty acids.—Much activity has continued in the synthesis of long-chain acids identical or akin to those which occur naturally. Synthetic production of numerous branched-chain saturated acids is much in evidence, as shown by recent issues of these Reviews. In this report reference can only be made, however, to one or two recently published syntheses of unsaturated long-chain acids which increase the already impressive list of these acids (including linoleic, oleic and elaidic, and *cis-* and *trans-* forms of other monoethenoid acids) which have now been synthesised in the laboratory by unequivocal methods. A simple synthesis of octadec-6-ynoic acid (which occurs naturally as tariric acid) has been given by Lumb & Smith (101). The lithium salt of *n*-tridec-1-yne, $CH_3 \cdot [CH_2]_{10} \cdot C{\equiv}CLi$, reacts with 1-iodo-3-chloropropane and the product, by malonic ester condensation, yields hexadec-4-ynylmalonic acid, $CH_3 \cdot [CH_2]_{10} \cdot C{\equiv}C \cdot [CH_2]_3 \cdot CH(COOH)_2$, which decarboxylates on heating and gives tariric acid. Reduction of the synthetic octadec-6-ynoic acid yielded, in addition to stearic acid, *cis*-octadec-6-enoic acid identical with natural petroselinic acid from Umbelliferous seed fats. Boughton, Bowman & Ames (102) have synthesised the *cis-* and *trans*-forms of myristoleic, palmitoleic, and gadoleic acids from acyloins ($R \cdot CHOH \cdot CO \cdot [CH_2]_n.COOH$) prepared from appropriate 2-hydroxy-saturated acids and the ester of heptane-1,1,7-tricarboxylic acid. The acyloins were reduced to a mixture of enantiomorphous dihydroxyacids which was resolved by crystallisation, the individual dihydroxy-acids being then converted to the corresponding dibromo-acids which were debrominated to give the respective *cis-* and *trans*-ethenoid acids.

A note on the preparation of symmetrical mixed triglycerides (notably β-oleodisaturatedglycerides) by Craig, Lundberg & Geddes (103) gives useful help in connection with the production of these materials for comparison with similar glycerides isolated from natural sources. Lutton (104), in his further studies of the polymorphism of α-oleodistearin, α-oleopalmitostearin, and α-oleodipalmitin and of β-oleopalmitostearin, emphasised that the highly characteristic differences in behaviour of the symmetrical and unsymmetrical oleo-glycerides should serve as a basis for identifying disaturated component glycerides of many natural fats.

It was stated at the outset of this review that the treatment would be selective, with the intention of surveying the progress made in a number of related lipid research topics in recent years. For this reason many interesting contributions of the past twelve months may have failed to receive mention. A more complete bibliography of currently published researches in the field is however available in the annual review by Piskur (105).

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NUCLEIC ACIDS, PURINES, AND PYRIMIDINES[1]

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Last year's edition covered only chemical and structural aspects, and this review emphasizes the developments of the last two years which are pertinent to the metabolic aspects of the problem. Qualitatively the picture of the biosynthesis of nucleic acids has developed appreciably, and the known precursors are being applied in numerous studies of tissue, bacterial, and virus nucleic acid synthesis. Much remains to be done to clarify the observations concerning alternate pathways possible at all stages of nucleic acid biosynthesis. The quantitative aspects of their metabolism are greatly complicated by these multiple pathways and by the necessity of working with "fractions" rather than with discrete components, and a healthy skepticism must be retained in interpretation of data obtained upon such materials.

Nucleic Acids of Tissues

Desoxyribonucleic acid (DNA) as a reference for expressing changes in tissue composition.—The practical utility of the concept of the constancy of the DNA content of the cell, derived from the work of Boivin, the Vendrelys, Mirsky, and of Mandel on the quantity, and of Chargaff & Mirsky on the composition, has been stressed by Davidson & Leslie (1 to 3). In most normal somatic tissues the average amount of DNA per cell is essentially constant, and, with proper caution, it may be used as a reference through which changes in other constituents may be expressed in terms of the amount per cell. It is similarly possible to use the increase of total DNA in a given specimen as a measure of cell multiplication. The precision of the methods upon which the "constancy" of DNA is based leaves something to be desired and variations of several per cent in the mean values remain experimentally undetectable (4, 5). Optical methods applied to individual nuclei continue to show (4 to 9) variations in the DNA values, or in some cases for pentose nucleic acid (PNA)[2] plus DNA values (10), with frequency distribution maxima at diploid (or polyploid) values. The extent to which these are due to errors inherent in the methods or to biological variations has not been decided. Chemical methods have shown the DNA per cell in a series of *saccharomyces* from haploid through tetraploid (11), and in haploid and

[1] The survey of the literature pertaining to this review was concluded in November, 1952.

[2] There is a growing number of identifications of ribose in PNAs, or in nucleotides therefrom, and no other sugars have recently been reported. However the total sugar has not been accounted for as ribose and numerous examples remain uninvestigated. The more conservative abbreviation PNA is used here, although, as has been pointed out repeatedly, in oral presentations the use of RNA minimizes confusion with DNA.

diploid *aspergillus* conidia (12), to be consistent with an approximate integral ratio in the polyploid series, to vary widely in certain salivary gland nuclei (13, 14), but to be at a constant high level in the mature *Drosophila* larvae salivary gland (i.e., giant chromosomes) (15). About twice the host diploid values were found in certain ascites tumors (8, 16) and in fowl sarcoma (17). Transplanted leukemic spleen nuclei (rapidly growing) were higher in DNA than spontaneous (slowly growing) ones (18). It is now generally agreed that the high values found for the DNA of liver nuclei are due to the characteristic polyploidy of hepatic tissue, and that higher than average values may be expected in rapidly dividing tissues. Campbell & Kosterlitz (19) have carefully confirmed their and Mandel's (20) (and others, see 19) earlier findings that there is no change in the average DNA content of liver cell nuclei of adult rats on protein-free diets, but Ely & Ross (21) and Lecomte & de Smul (22) each reported a significant increase in the DNA content per liver cell in young rats receiving protein-depleted diets.

Despite the variations and the frequent need to resort to cell counts for the standardization of the tissue being studied, it appears that the DNA content of the normal cell is probably the most constant dimension among variables such as total nitrogen, weight, etc. which can be used as a standard upon which to base other analytical values in terms of the amount per cell. In a few studies, use has been made of the DNA value as a reference for changes in other components (15, 23 to 29), and there are a number of other studies in which such a presentation of the data might be advantageous.

Composition of nucleic acids.—A primary development in the picture of the species specificity of composition of DNA [Chargaff *et al.* (30, 31, 32; Smith, Wyatt *et al.* (33, 34)] is the presence of small amounts of 5-methylcytosine in DNA of mammals (34, 35), plants (35), and hens' eggs (100), but not in DNA preparations of microbial (33), rickettsial (36), protozoal (37), phage (33, 38), or viral (33) origin which have been investigated. To fit those DNAs containing 5-methylcytosine into the "AT" or "GC" types of Chargaff (31), it was necessary to consider 5-methylcytosine and cytosine together (31, 39). The nucleoside (40) and a nucleotide (41) of this pyrimidine have been characterized. Mirsky (42, 43) has deduced a decreasing DNA content of the cell in the course of the evolution of vertebrates, and Mandel (44) has noted a considerable similarity of the DNA content of the leucocytes of several mammals.

Variations in the compositions found for the different PNA preparations from the same source (45, 46) can be attributed to the present inability to prepare representative PNA samples, and resort must be made to direct analyses of the bases in a tissue sample. Fairley, Seagran & Loring (47) have reported variations in composition of the PNA components in various cell fractions, when determined by differential spectrophotometry of binary mixtures of the purines and of the pyrimidine nucleosides (46). Their procedure differs primarily from that of Kerr *et al.* (45) in that the pyrimidine nucleotide mixture is enzymatically dephosphorylated and purified with ion ex-

change resin. Davidson (3) has also reported compositions of the PNAs from cell fractions, based upon ionophoretic separation of the nucleotides (48), which indicated minimal differences between the cytoplasmic fractions, but these were considerably different from the nuclear PNA. Variations in the relative renewal of the phosphorus of the individual nucleotides of the cytoplasmic PNAs (49, 50) attests to their metabolic dissimilarity. A difference in the enzymatic degradation of the PNAs of the tobacco-leaf chloroplasts and sap suggests their nonidentity [Parker (51)]. No differences were found in the composition of the PNA of yeasts grown in different media [Thomas (52)].

Factors which influence the nucleic acids of tissues.—Reference is made here to studies bearing on attempts to elicit a satisfactory relationship of PNA to some metabolic or functional process.

In several organs of the chick embryo Leslie & Davidson (24) found that the rates of increase of acid soluble phosphorus and PNA paralleled one another, as did the amounts of lipid-P and protein-N. In chick heart explants cultured *in vitro*, those authors (23) also found that protein and lipid-P first increased followed by increases of PNA, DNA, and acid soluble phosphorus coincident with cell multiplication. In these experiments, as in others with the chick embryo (53, 54), bone marrow cells (55), and in *Staphylococcus muscae* (56), the increased ratio of protein-N to PNA is characteristic of more mature cells.

Analyses for PNA, DNA, and the individual bases were carried out on the growing chick embryo [Reddy *et al.* (54)], and, although not in full agreement with the more extensive study of Novikoff & Potter (53), these were purported to show a parallelism of protein and PNA synthesis. At all stages the amount of PNA in the chick embryo was greater than that of DNA, whereas in the mouse embryo there was nearly twice as much DNA as PNA (57).

There was an increase of DNA as well as of PNA (in terms of amount per gm.) in the liver, lung, kidney, and lymph nodes of tumor-bearing or pregnant rats or mice [Cerecedo *et al.* (58, 59, 60)]. Payne *et al.* (61, 62) found increased incorporation of P^{32}, formate, and glycine into the DNA of liver, and in some cases into the spleen and kidney, of tumor-bearing or pregnant animals. McIndoe & Davidson (17) found the same in tumor-bearing fowl, and these results suggest stimulation of DNA production in organs of the host.

Increases in nucleic acid content of tissues were accompanied by increases in purines [Lombardo *et al.* (60)], with that of guanine being greater than that of adenine in liver, lung, and lymph nodes. Since it was the adenine and guanine of the total tissue sample which was measured, an increase of an individual nucleic acid high in guanine could account for the results. In the chick embryo (54) there was no increase in thymine between the 7th and 12th days when total DNA was increasing, but one hesitates to accept this alone as evidence of a changing composition of DNA.

Possible correlations between the incorporation of glycine into the proteins and the nucleic acid bases under various conditions of growth is the subject of a broad study by Hammarsten and co-workers (63). They showed (64) that an increased renewal of PNA, but not an increase in quantity, is associated with protein synthesis in yeast. Eliasson *et al.* (65) found a direct correlation between the extent of incorporation of glycine-N^{15} into the protein (66) and the purine bases of regenerating (after phenylhydrazine poisoning) bone marrow of hens. Under similar conditions in rabbits Holloway & Ripley (67) find that the degree of incorporation of leucine into the protein could be correlated with the PNA content of the reticulocytes, and Lovegrove, DeLuca & Rossiter (68) find the incorporation of P^{32} to be correlated with the percentage of nucleated reticulocytes. A large number of experiments (63, 65) performed at various time intervals after partial hepatectomy of rats yielded data on the synthesis of several components at various stages of regeneration of liver. The greatest incorporation of glycine into the purines of both PNA and DNA occurred at about 30 hours and into glycine and other amino acids of the protein at about 60 hours. The greatest incorporation of P^{32} into the PNA was also found to occur at 20 to 30 (69) or 16 to 24 hours (70) post hepatectomy. The maximum incorporation of glycine into the purines coincided with the maximum increase in the quantity of the polynucleotides and with the maxima of mitosis and of the size of the nuclei and nucleoli (63). Tyner *et al.* (71) report that the activity of glycine-2-C^{14} can be detected in the protein fraction before it can be detected in the nucleic acids. Although evidence of some correlation of the times and extents of the renewal of PNA and protein continues to accumulate, no functional interrelationship can yet be implied.

Price (56) studied resting cells of *S. muscae* and found that the PNA to protein ratio, but not the PNA content, was always proportionate to the growth rate. In cells adapting to lactose there was an initial temporary loss of PNA, but there was never an increase in protein without a simultaneous increase in PNA. However, in the case of virus protein, the work of both Cohen *et al.* and Evans *et al.* has demonstrated that in virus infected bacteria this protein can be synthesized without any increase or turnover of the PNA of the host cells. In the mammal Abrams (72) found that x-ray irradiation reduces the incorporation of glycine into PNA purines but not into protein, similar to the effect of x-ray on the incorporation of carboxyl-labeled acetate into DNA and protein (73) or of carbon dioxide into mixed nucleic acids (74).[3]

[3] The technique of roller tube tissue culture has been used by Gerarde, Jones & Winnick (75) for study of the incorporation of C^{14}-amino acids and P^{32} into chick embryo tissue explants and, peculiarly, maximal incorporation of P^{32} into PNA and DNA occurred in media (embryo extract) favorable to growth, while maximal incorporation of amino acids occurred under conditions (Tyrode's medium) where the tissue was undergoing autolysis. Brunish & Luck report the "incorporation" in a firmly bound manner of amino acids into purified DNP or histone, under *in vitro* conditions which could not be considered to be enzymatic (76).

Jeener (77) found no regular relationship of PNA with protein-N or with growth rate in *Polytomella coeca*. After growth was limited by phosphate depletion, the addition of phosphate resulted in an initial decrease of PNA but increase of protein. Studies with P^{32} led to the hypothesis that, in any cell fraction, the quantity of PNA synthesized is proportional to that present. An independent autocatalytic reduplication of each class of cytoplasmic particles was proposed. This contraindicates the previous hypothesis from that laboratory that small cytoplasmic particles "grow" into larger ones [Chantrenne (78)], as do the observations that P^{32} incorporation into the smallest particles of liver cell fractions may be less than into larger ones (50, 79).

Thus no concrete evidence has come forth to clarify the interplay between PNA and protein, and sufficient evidence is accumulating to question the hypotheses of Caspersson & Brachet that PNA is primarily involved in protein synthesis. The inability to satisfactorily subdivide the cytoplasmic PNAs (or nucleoproteins) into characterized fractions may be obscuring vital evidence, and the present data may require reappraisal at some future date.

Several recent suggestions of other roles for PNA have been made. Binkley (80, 81) has claimed the isolation from pig kidney of a PNA preparation with cysteinylglycinase activity, but experimental details have not been presented. However, Chantrenne (82) finds no correlation between the sedimentation of PNA and this peptidase. Heppel & Hilmoe (83) again confirm that a polynucleotide fraction from PNA can induce the formation of a hemolysin by *Staphylococcus pyogenes* and found no mono or dinucleotide which was effective. The high PNA content of secretory cells has long been reported (84, 85), and in the *Drosophila* salivary gland Lesher (86) has shown that the PNA content is high during the secretory phase but drops during the growth phase. Leuchtenberger & Schrader (13) found 30-fold variations in the DNA content of *Helix aspera* salivary gland nuclei and correlate the high DNA with production of secretory granules.

In mouse pancreas, Rabinovitch *et al.* (87) find no changes in PNA content after pilocarpine and suggest that earlier histochemical studies merely measured redistribution of polynucleotides. Guberniev & Il'ina (88) have found that *in vivo* stimulation of secretion (by pilocarpine, and also by bile in the case of liver) greatly increases the incorporation of P^{32} into the PNA of parotid glands, liver, and pancreas. The PNA is elevated in the secretory portion of the silk glands of *Bombyx mori* (89). From such data it is difficult to divorce the synthesis of the protein secreted from the process of secretion, but Hokin (90) has used pigeon pancreas slices and found an increase in P^{32} uptake in PNA when secretion of amylase is stimulated by carbamylcholine, but no increase when synthesis of the enzyme is stimulated by an amino acid mixture.

Munro, Naismith & Wikramanayake (3, 91) report an intriguing observation. With rats on high protein diets, an increase in calorie intake caused a rise in the total amount of PNA without altering the percentage of P atoms exchanged, whereas on a protein-free diet, calorie intake produced a minimal

change in total PNA but an increase in the percentage of P atoms exchanged. It was suggested that the amount of P^{32} incorporated was related to energy intake, and it was reported (92) that the renewal of the PNA of the nuclear, mitochondrial, microsomal, and supernatant fractions were influenced in that order. Such observations are reminiscent of earlier speculations regarding PNA as an intermediary in energy storage and utilization (93, 94).

Bartholomew & Mittmer (95) review the chemical theories of gram staining and discount any specific role of Mg-PNA, despite earlier proposals. The yeast growth stimulating activity produced by irradiation of yeast, and once (96) attributed to nucleic acid derivatives, has now (97) been shown to parallel amino-N, and not ultraviolet absorbing material. Injection of commercial yeast nucleic acid and an antigen are reported to stimulate total serum globulin production more than either alone (98). In the case of possible roles for DNA it has now been shown that the DNA present in some forms of avidin plays no role in the biotin binding properties (99, 100); and the claim that DNA fulfills the requirements for a coenzyme of β-glucuronidase (101) has been disputed (28, 102).

Attention should be drawn to studies of the influence of the nucleus on the metabolism of the cytoplasm in Brachet's laboratory (103), and of the enzymes of nuclei isolated in nonaqueous media in Mirsky's laboratory (104).

Influence of various agents on nucleic acids of tissues.—Increases in PNA content, usually paralleled by increases of other substances and frequently associated with cell hypertrophy, continue to be associated with the action of agents which cause a stimulation of anabolic processes, such as estrogens (105, 106), testosterone (106), insulin (107), and methylthiouracil (108, 109). Conversely, thyroxine decreases the PNA content of the thyroid (109). Cortisone increases the output of urinary purine (110), and reduces the incorporation of formate into nucleic acids (111); it also reduces the protein content and increases the ratio of PNA to DNA in rabbit (112) and guinea pig (113) livers. The latter results were interpreted as a preferential effect on the catabolism of DNA, but the determination of the DNA per cell is necessary before the conclusion can be accepted.

The administration of vitamin B_{12} enhances the liver protein, PNA and DNA in terms of mg. per unit of body weight [Sahasrabudhe & Rao (114)], but Rose & Schweigert (26) showed that, while the amounts per gram of liver PNA and DNA were increased by supplementation of B_{12} deficient animals, the amounts per cell were not altered. They also found that the renewal (by glycine-N^{15}) of both protein and PNA was enhanced with the B_{12}. The PNA content of nerve tissue (115) is enhanced by B_{12} administration, and the uric acid content of chick blood is once reported to be increased (116) and once to be inconsistently affected (117). A dual role of B_{12} in nucleic acid biosynthesis (118) has been confirmed by Weygand, Wacker & Wirth (119) who demonstrated that various purines and pyrimidines and desoxyguanosine were required to replace B_{12} for *Lactobacillus leichmannii* 313, and Downing,

Rose & Schweigert (120) showed the same with various bases plus thymidine for *L. leichmannii* 313 and 327.[4]

Pyridoxine deficiency coupled with desoxypyridoxine administration led to a decrease in the DNA of spleen (but not of other organs), and a specific role of B_6 in nucleic acid synthesis was suggested [Cerecedo *et al.* (121)]. However, protein deprivation had been shown to result in a loss of as much as 50 per cent of the DNA and PNA of the spleen (completely reversible on administration of protein) [Jacob, Mandel & Mandel (122)], and it seems probable that the B_6 deficient animals had sufficient disruption of their protein metabolism to make it unnecessary to invoke a specific role of B_6. Vitamin E deficient rabbits showed an elevated muscle PNA and DNA, and an increased allantoin output, which could be counteracted by α-tocopherol administration (123). It is claimed (124) that ascorbic acid is involved in DNA formation. Pentobarbital reduced P^{32} incorporation into the DNA of spleen and bone marrow (125).

The reduced PNA content, of the whole cell or the nuclei, of the megaloblasts of megaloblastic monkeys was returned to normal within 12 hours after folic acid administration; there was also a greatly increased incorporation of P^{32}, particularly in the nuclear PNA [Lowe & Barnum (126)]. When folic acid is administered to humans there ensues a uricaciduria (127). The action of the folic acid antagonist aminopterin resulted in an increased PNA to DNA ratio in tumors (128), which was said to be due to a lowered DNA content similar to that observed in folic acid deficient *L. casei* (129). Folic acid supplementation partially reverses (130) the amethopterin inhibition (131) of formic acid-C^{14} incorporation into crude nucleic acid fractions. That folic acid deficiency results in an equal decrease in the incorporation of formate into both positions 2 and 8 of the purines was shown by Drysdale, Plaut & Lardy (132). By a double tracer experiment, it was found that the influence of aminopterin was to reduce the synthesis *de novo* of purines and thymine (from formic acid-C^{14}) but not the synthesis of nucleic acid per se (from adenine-1, 3-N_2^{15}) [Goldthwait & Bendich (133)]. Although folic acid deficiency reduced the incorporation of formate into the nucleic acids in liver, and not in the remaining viscera (132), the aminopterin reduced the incorporation into those of small intestine and spleen as well (133).

Intermediary Metabolism

Origin and metabolism of pentoses.—The origin of pentose from phosphogluconate [Dickens (134)] has received renewed attention. Cori & Lipmann reported that the conversion of 6-phosphoglucose to 6-phosphogluconate proceeds via 6-phosphogluconolactone (135). The work of Scott & Cohen (136, 137) and of Horecker & Smyrniotis (138) in elucidating the conversion, by

[4] Weygand *et al.* (119) report that *L. leichmannii* 313 is inhibited by adenine (and this inhibition is blocked by guanine), while Downing *et al.* (120) report that 327 (but apparently not their 313) is so inhibited.

enzymes from *Escherichia coli* or yeast, of 6-phosphogluconate, via 3-keto-6-phosphogluconate, to a mixture of ribulose, ribose, and arabinose-5-phosphates were reviewed in 1951 (139, 140). While extracts of mammalian tissues had been observed to catalyze the reduction of triphosphopyridine nucleotide by phosphogluconic acid (141, 142), it is only recently that an enzyme from mammalian tissue has been demonstrated to accomplish the above conversions [Seegmiller & Horecker (143)].

There are new examples of cleavage of pentoses to 2 and 3-carbon products (144 to 148). Such a cleavage of desoxyribose did not appear to occur (149), although it now (150) seems that desoxyribose-5-phosphate is cleaved to a triose and acetaldehyde and that it was inhibition, by phosphate, of the transformation of desoxyribose-1 to the 5-phosphate which affected the earlier picture. The possibility of pentoses arising by a condensation of 2 and 3-carbon units receives tenuous support from the observation that an enzyme from *Micrococcus pyogenes* can lead to the condensation of glycolaldehyde and triosephosphate to a xylulose-phosphate [Marmur & Schlenk (151)]. Enzymes of bacteria (148, 152), and mammalian tissues (148, 153) will bring about an aldol condensation of acetaldehyde and glyceraldehyde phosphate to desoxyribose phosphate, and Racker (148) has emphasized that, with acetaldehyde present, the latter enzyme plus enzymes which cleave ribose to triosephosphate (155) could permit the conversion of ribose to desoxyribose.

The hexose phosphates which can be formed from ribose-5-phosphate by a series of factors from rat liver [Dickens & Glock (156, 157)] cannot arise via trioses and the Embden-Meyerhof scheme since fructose-1,6-diphosphate will not serve as an intermediate (157, 158). However Horecker & Smyrniotis (158, 159) have isolated sedoheptulose from such a system and present evidence that this 7-carbon sugar (which has also been found among the products of photosynthesis) arises from condensation of triosephosphate and a tetrose presumably derived from two 2-carbon moieties of pentose. They speculate that hexose may arise from a 7-carbon sugar in a manner similar to that of 5-carbon from 6-carbon sugars.

A conclusion as to which of the potential pathways furnishes the principal source of mammalian nucleic acid pentoses has not been reached. Gluconic acid-C^{14} carbon appeared in the total crude rat nucleic acid fraction (160), but studies of the patterns of incorporation of acetate-C^{14} or formate-C^{14} into glycogen and PNA-ribose exclude the conversion of hexose to ribose as a major source of ribose [Bernstein (161, 162)], and leave open the possibility of a condensation of 2 and 3-carbon units. With acetic acid-α-C^{13} and carboxyl-C^{14} it was shown that acetate was not incorporated into ribose as a 2-carbon unit [Low (163)]. Glycine-carboxyl-C^{14} was incorporated into ribose (164). From the fact that cytidine leads to both PNA and DNA pyrimidines (165), and that free cytosine is probably not an intermediate, it has been suggested that desoxyribose may arise from ribose while the latter is in ribosidic linkage. It has also been demonstrated that in the rat some C^{14} of the ribose of a pyrimidine riboside may appear in the desoxyribosides (166).

Until further details appear it seems possible that the incorporations observed could be the result of transglycosidation (167) of the pyrimidine, and of C_3-C_2 cleavage of the ribose with partial reutilization of the C_3-unit in synthesis of the desoxyribose. From studies with glucose-1-C^{14} in *E. coli* (168) it appeared that the bulk of the PNA-ribose arose via the phosphogluconate pathway with loss of the C_1, while much of the C_1 of the glucose reached the bacterial or phage DNA-desoxyribose.

By means of an assay specific for desoxyribose in any type of nucleosidic link (169) a large "store" of such desoxyribose was detected in the frog egg [Høff-Jorgensen & Zeuthen (170)]. Similarly large amounts of guanine and hypoxanthine (171) in the frog egg, and of thymine in the sea-urchin egg (172), were found on analysis and assay respectively.

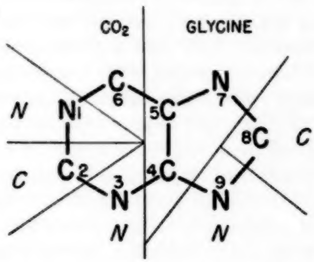

FIG. 1. Origin of the atoms of the purine ring.
C— from 1-carbon sources (formate, formaldehyde)
N— from NH_3 (via aspartate, glutamate, glutamine)

Synthesis of purines de novo.—The sketch indicating the source of the atoms of the purine skeleton, first used in these reviews in 1949, is modernized in Figure 1. The erroneous report that acetate was a precursor of carbons 2 and 8 has been corrected (173, 174, 175). The incorporation of formic acid has been repeatedly verified, and it has been shown to be essentially equal in carbons 2 and 8 of tissue nucleic acid purines (132, 174, 176)[6]; formaldehyde (176, 177) and the amidine carbon of histidine (178) are also utilized to form these carbon atoms. The nitrogen of aspartic acid, glutamic acid, and glutamine proved more effective than ammonia as sources of the 1, 3, and 9 nitrogens in a pigeon liver preparation (179, 180), but *in vivo* (pigeon) the behavior of the N^{15} of aspartic acid was similar to that of an ammonia source (181). Various portions of this biosynthetic pattern have been repeatedly confirmed for the excretory purine, polynucleotide purines, and soluble nucleotide purines in several species. Dimroth *et al.* (182) find that

[6] Drysdale *et al.* (132) comment that, although formic acid may be oxidized to carbon dioxide, little incorporation into the 6 position of purines was found. However, it now appears that the extent of oxidation of formate is heavily dependent upon the size of the administered dose, and they administered small quantities.

serine-β-C^{13} leads to more label in the 6 than in the 2 and 8 carbons in *Torula*. They propose that the 4, 5, 6, 7 atom unit arises directly from serine, but this might be peculiar to the versatile synthetic capacities of *Torula*.

More detailed reports on the origin of hypoxanthine in pigeon liver preparations have demonstrated that inosinic acid (IMP) is the first purine derivative produced and that inosine and hypoxanthine are derived therefrom [Greenberg (177)]. The production of free 4-amino-5-imidazolecarboxamide (carboxamide) could not be detected in this system (175, 177). But, when added, it (175) or hypoxanthine (183) can lead to IMP, and when the carboxamide is being utilized it apparently must be combined with ribose and phosphate prior to completion of the purine ring system. It was noted [Schulman & Buchanan (184)] that the incorporation of formate into the 2 position of IMP (but not of inosine or hypoxanthine) could far exceed the incorporation of glycine into the 4,5-positions, and this was attributed to an equilibrium reaction between IMP and a carboxamide-pentose-phosphate plus formate. Later Buchanan reported (180) that omission of carbon dioxide from the medium greatly reduces the extent of synthesis *de novo* and thus permits exchange at the 2 position to predominate. Greenberg (185), using an inhibited *E. coli* system, reported the detection of a pentose derivative of the carboxamide, and possibly of its phosphate. Ben-Ishai *et al.* (186) present evidence of the incorporation of the carboxamide into desoxyribosidic linkage by *E. coli*. An effect of methionine in preventing accumulation of the carboxamide [Bergmann *et al.* (187); Gots & Chu (188)] has been considered in terms of methionine as the precursor of the 2-carbon, but in view of the similar activity of ethionine (187) and the knowledge of an "active methionine" (189) the alternative of a catalytic function (185) would seem more attractive. Stewart & Sevag (154) showed that carboxamide accumulation was favored by anaerobic metabolism of glucose, but that aeration blocked its accumulation.

There was no evidence of the formation of soluble adenine derivatives by the above pigeon liver preparations (177), although in rat liver cells (190) and yeast (191) they arise from small precursors. They can arise from added adenine in a pigeon liver preparation (192), but comparison of the types of homogenates used in the two laboratories is not yet possible. From the available data it is not yet possible to decide where inosinic acid lies in relation to the normal pathway of synthesis of other purine derivatives. Neither can the mechanism of utilization of the carboxamide,[6] hypoxanthine, or adenine in this system, nor of administered free purines by intact organisms, be particularized; the known nucleosidases or transglycosidases can account for introduction of a purine into glycosidic linkage, but they cannot account for net synthesis of nucleotides unless a source of the pentose is available.

Utilization of purines by various species.—In organisms which do not require preformed purines the utilization of exogenous purines represents a

[6] The carboxamide, but not the 4-hydroxy analogue, can also be utilized by the rat (193).

by-passing of a portion of the usual pathways of synthesis *de novo*. The use of isotopically labeled purines permits a direct demonstration of their utilization, and also a direct measure of the extent to which they are transformed into derivatives of other purines. Different species present a whole spectrum of variations in their pattern of purine utilization and interconversion.

A reinvestigation of the utilization of guanine by the Sherman (194) and Sprague-Dawley (195) rats showed that small incorporations of guanine-8- or -2-C^{14} could be detected, although use of guanine-1,3, 2-amino-N^{15} had failed to demonstrate it. The C57 mouse, in which a more significant utilization of the guanine-N^{15} had been previously observed, was also shown to utilize adenine and to convert it into polynucleotide guanine (194). In rabbit bone marrow slices there was a small conversion of adenine into guanine and vice versa, and of each into soluble hypoxanthine; all three could also reach the polynucleotide purines (196).

A variety of microorganisms studied include *Saccharomyces cerivisiae* (197) and *Aerobacter aerogenes* (198) which readily utilize adenine and convert it into polynucleotide guanine, but also use guanine. A haploid *S. cerivisiae*, and a mutant thereof, can convert adenine of the medium into PNA guanine to a greater extent than into PNA adenine (199). Other species range, with increasing conversion of guanine into adenine, through *Paramecium aurelium* (killer and sensitive strains significantly different) (198), *E. coli* (200, 201), and a chrysomonad *Ochromonas* (198), to *L. casei* which presents the maximum conversion of each into the other (202). The opposite extreme in this comparative biochemistry of purine utilization is represented by *Tetrahymena geleii* (203) and *L. leichmannii* (204) which use guanine and convert it into polynucleotide adenine but use adenine for polynucleotide adenine synthesis only. The facility of interconversion also varies; in yeast, that of adenine to PNA guanine is "spared" by guanine (197); in *L. casei* when both purines are present nearly half of each is converted into the PNA derivative of the other; in *L. leichmannii*, that of guanine to PNA adenine is "spared" by adenine (204). In seven instances tested, the fate of 2,6-diaminopurine is qualitatively similar to that of guanine, including conversion into adenine, and in *L. casei* 2,6-diaminopurine can be utilized to the same extent as can adenine or guanine. It might be provisionally inferred that in synthesis *de novo* the pathway leading first to adenine and thence to guanine may predominate in some species, while that leading to guanine and thence to adenine may predominate in others.

All evidence has indicated that, once formed, the purine ring remains intact. The nonincorporation of formate-C^{14} into polynucleotide purines in folic acid deficient medium, the results with various purines labeled in the 2 or 8 position in *L. casei* (202), the parallelism of the incorporation of both isotopes of adenine-1,3-N^{15}-8-C^{14} in the rat (205) and of guanine-2 or -8-C^{14} in *L. leichmannii* (206), the nonincorporation of medium $N^{15}H_3$ into the ring nitrogens of administered purines in *E. coli* (200), all attest to this. Investigations of possible labilities of the purine ring in polynucleotide purines with formate

in the pigeon (176) and in folic acid deficient rats (132), and with glycine in the mouse (207), did not indicate any unusual lability of individual positions. A discussion of a possible lability of position-2 of diaminopurine (208) involved interpretations which the original authors (209) did not feel were justified by the data available. When adenine-6-C^{14} and $N^{15}H_3$ were administered to *E. coli* there was also little exchange of the amino group in the adenine utilized (200).

With *L. casei* in folic acid containing medium, the amount of PNA purines derived by synthesis *de novo* is approximately inversely proportional to the concentrations of the purine furnished, except at very low levels (202), and a preferential utilization of administered purine was also observed in *E. coli* (200, 201). In the rat the administration of adenine at 1.9 mM per kg. suppressed the incorporation of glycine, particularly in the liver (195), and 0.2 mM suppressed the incorporation of formate, although the effect of 0.2 mM or less was negligible (133).

With varying quantities of labeled adenine administered to rats (from 0.02 to 1.5 mM per kg. per day), the dilution factors encountered, because they are not inversely proportional to the dose, suggest a mixing with an appreciable quantity of "available purine" (205). Quantitative variations in utilization by individual animals prevent satisfactory calculations of the size of this "pool", but it is of the magnitude of 20 to 30 mg. per kg. per day as estimated from the dilution encountered in the direct oxidation of adenine to 2,8-dioxyadenine (210).

Adenine-4,6-C^{14} administered to the mouse was almost immediately incorporated into the acid soluble fraction and 20 per cent of it was still in that fraction after 24 hours [Bennett (211)]. Similar results were obtained in the rat [Marrian (212)]. In pigeon liver homogenates, the adenine-8-C^{14} present was rapidly incorporated into soluble adenine nucleotides (and hypoxanthine), and the presence of an adenosine phosphate other than adenosinemonophosphate, adenosinediphosphate, or ATP was detected [Goldwasser (192)]. The incorporation of adenosine-8-C^{14} into the total soluble adenine of rat muscle was 2.5 times that into the ATP (and adenosinediphosphate) fraction [Lowy *et al.* (213)] and these observations, plus chemical evidence [Snellman & Gelotte (214)] indicate the existence of uncharacterized adenine derivatives in soluble nucleotide fractions of tissues.

The renewal of the *a* and *b* isomers of the purine nucleotides obtainable from PNA (215) has always been found to be essentially the same whether the precursor be phosphate (216), formate (217, 218), adenine (205), nucleosides (213), or nucleotides (219), and this is in agreement with chemical evidence concerning their origin (220, 221).

Origin of pyrimidines.—A concise diagram outlining pyrimidine origin is not yet possible. The methyl group of thymine has now been found to originate from formate (217), but a failure to detect incorporation of the α-carbon of glycine is reported (222). There is much additional evidence that orotic acid or its derivatives are involved in the synthesis *de novo* of pyrimidines,

and samples isotopically labeled in the 1,3; 2; or 6-positions have been shown to be utilized by rat (223) and human (224) tissues, *E. coli* (225), and *L. bulgaricus* (226a, 226b).

The origin of orotic acid is receiving much attention. A riboside of orotic acid has been shown to be produced by a pyrimidineless *Neurospora* (227). The orotic or ureidosuccinic acid requirement for an *L. bulgaricus* has been shown to be very specific (226b) and both orotic acid-2-C^{14} and ureidosuccinic acid-ureido-C^{14} have been shown to be pyrimidine precursors [Wilson (228); Wright *et al.* (226a)]. Aspartic acid-β-C^{13},γ-C^{14} and -amino-N^{15} was tested in rat liver slices and its methylene carbon, and less extensively the γ-carboxyl, reach the pyrimidines, although the amino group was but poorly utilized [Lagerkvist *et al.* (229)]. This result in the rat is not consonant with the inference that ureidosuccinic acid may furnish the complete pyrimidine skeleton in *L. bulgaricus.*

In connection with a study of orotic acid-6-C^{14} incorporation into cell fragments at various time intervals, Hurlbert & Potter (230) report a slightly greater conversion to PNA cytosine, in contrast to the original findings of Arvidson *et al.* (231) who found slightly greater conversion to uracil. Hurlbert (232) also reports the detection of a highly labeled uridine phosphate in the acid soluble fraction of liver at short times after administration of orotic acid-6-C^{14}. Reichard (233) incubated rat liver slices with $N^{15}H_3$ and a bank of nonlabeled orotic acid and found more extensive incorporation of the isotope into the orotic acid than into the polynucleotides. He proposes that orotic acid is a normal intermediate, but the great lability (227) of the glycosidic linkage in orotidine might have permitted the liberation of the free orotic acid found. However, from the failure of uracil to be utilized by the rat it would be presumed that administered orotic acid must proceed to at least a riboside before it is decarboxylated. If free orotic acid is a normal intermediate in pyrimidine biosynthesis, this is another difference from the scheme of purine biosynthesis, where the ribose is apparently attached before completion of the purine ring.

Utilization of nucleosides and nucleotides.—Several synthetic purine nuclosides (234), containing C^{14} in the purine moiety, have been studied in the rat [Lowy *et al.* (213)]. Administration was by intraperitoneal injection and conditions (0.2 m*M* per kg., three days) were directly comparable to those under which most purines and nucleotides have been studied, so some comparison of the series is possible. Adenosine-8-C^{14} was incorporated into PNA adenine and guanine and 2,6-diamino-9-β-D-ribofuranosylpurine-2-C^{14} into PNA guanine, but in each case to an extent less than half that of the parent purines. Inosine-8-C^{14}, in contrast to hypoxanthine, did lead to some renewal of both PNA purines. Guanosine-2-C^{14} and crotonoside-2-C^{14} (isoguanineriboside) each led to a trace renewal of the PNA guanine of the order found with guanine-8-C^{14}, although it had not been detectable with guanosine-N^{15} (235). When the adenosine (or inosine) was administered with large quantities of unlabeled adenine, little isotope appeared in the 2,8-dioxyade-

nine deposited in the kidneys, which suggested that the nucleoside is not utilized via free adenine. The adenosinekinase, demonstrated by Caputto (236) and Kornberg & Pricer (237), which will phosphorylate adenosine or the riboside of diaminopurine in the 5 position, offers one possible anabolic pathway for these nucleosides.

With biosynthetically prepared purine nucleotides (N^{15} labeled, mixed isomers) it was found [Roll & Weliky (238)] that adenylic acid led to both PNA adenine and guanine, but that guanylic acid, in contrast to guanine or guanosine, led to a considerable renewal of PNA guanine. The sum of the PNA guanine derived indirectly from adenylic acid and directly from guanylic acid equalled the renewal of the PNA adenine from adenylic acid, and now explains the earlier observation that the PNA purines were equally labeled after injection of a mixture of yeast nucleic acid nucleotides. The individual isomers of the purine nucleotides, prepared from yeast grown with adenine-8-C^{14} (197), revealed that, in the rat the *a* or *b* isomers of adenylic acid each led to the same renewal of PNA purines as did adenosine (219). Since guanylic acid is the only guanine-containing compound incorporated to an appreciable extent, it appears that it may be involved in, or be in equilibrium with an intermediate on, a pathway leading to PNA guanine.

Cytidylic acid-N^{15} and uridylic acid-N^{15} were utilized (219) as precursors of polynucleotide pyrimidines to an extent almost identical with that to which the corresponding N^{15}-labeled nucleosides had been found to be utilized by the rat (239), and in each case there is some transformation into derivatives of the other pyrimidine. The utilization of the cytosine derivatives is approximately 10-fold that of the uracil derivatives and is of the order found with free adenine. With all of the pyrimidine derivatives there was a definite renewal of the DNA pyrimidines (219, 239). Some pyrimidine nucleoside nitrogen was found in the DNA purines (239), but with the nucleotides this was not observed (219).

Reichard & Estborn (240) have also shown that desoxyinosine is not utilized. Small amounts of desoxycytidine are incorporated as DNA cytosine and thymine, and thymidine leads only to thymine; they thus conclude that conversion of desoxycytidine to thymine must be irreversible in the rat.

Recently, Rose & Schweigert (166) have reported the first studies with a nucleoside labeled both in ribose and base. In the rat cytidine was incorporated intact as PNA cytidine, but little of the ribose accompanied that transformed into PNA uridine. However, in *E. coli* they found that most of the pyrimidine was incorporated without the ribose.

L. casei is a microorganism on which extensive studies of purine derivatives have been conducted (241 to 244). This organism shows the most extensive interconversion of the purines (242), including the formation of both adenine and guanine from 2,6-diaminopurine when the inhibitory action of the latter is relieved (202). A study was made of the relative utilization of a series of labeled purine derivatives in competition with synthesis *de novo*. The results of incorporation experiments in folic acid media paralleled those

of growth studies in folic acid free media. In general the purine moieties of the series of nucleosides were considerably less extensively utilized than were the corresponding free purines, and there was a less facile interconversion of the bases. Also there was a utilization of 2,6-diamino-9-β-D-ribofuranosylpurine under conditions where the free base was not utilized, and these facts suggested that the nucleosides are not utilized via the free purines, nor vice versa (243). In *L. casei* the incorporations of the *b* isomers of adenylic or guanylic acids were equal to those found for the free purines, although there was insignificant utilization of the *a* or the 5-isomers (244). The extent of utilization of the *b* isomers of the purine nucleotides is compatible with an assumption that in *L. casei* they could be one of the first products derived from the purines. However, the extreme differences between the responses of the rat and *L. casei* to the isomeric purine nucleotides permits no generalization. In yeast (197) the nucleosides and nucleotides were utilized far less extensively than were the parent purines.

A specific utilization of certain dinucleotides was shown by Merrifield & Woolley (245). *L. helveticus* 355 required uracil (or partially hydrolyzed PNA), and only dinucleotides with a terminal cytidine-5′-phosphate moiety would also serve. The specificity of the requirement here and for the *b* purine nucleotides in *L. casei* (244) would seem to exclude the factor of permeability as an explanation for either of these results.

Renewals of nucleic acids.—Experiments in which a variety of precursors have been used have led to varied results and divergent interpretations. Recent developments partially clarify some apparently conflicting evidence and at the same time increasingly reveal the complexity of the problem.

The observations of Hevesy, Marshak, and Brues that the majority of the incorporation of P^{32} into DNA occurs during mitosis continue to be confirmed (17, 246, 247, 248). Pelc & Howard (248) summarize the autoradiographic technique by which they conclude that DNA synthesis (in bean roots) occurs during interphase and ceases a few hours before prophase, and that those cells which cease dividing and which differentiate no longer synthesize DNA. In a patient treated with therapeutic doses of P^{32} there was no P^{32} detected in the DNA of brain, skeletal muscle, or cartilage, although there was considerable P^{32} in the DNA of liver and kidney and even more in the nodes and spleen [Osgood *et al.* (247)]. The incorporation of formic acid-C^{14} into the DNA purines and thymine in a series of organs of rats can also be approximately correlated with the degree of mitosis (133, 249); there is considerable incorporation into the DNA of intestine and spleen, small amounts into testis, liver, and kidney, and negligible into brain. The incorporation of P^{32} (61, 250) and of adenine-N^{15} (133) into the DNA of several of these organs in rats is also in this sequence. In strain A mice the incorporation of P^{32} into spleen DNA was greater than into intestine (61), however the physiological condition can greatly influence the renewal of DNA in lymphoid tissues (247, 251). More detailed comparison of the different precursors is not justified since some of the measurements involved the total

DNA and in others individual nucleotides of DNA are found to be renewed at different rates. Technical difficulties due to the low incorporation values as well as diverse experimental conditions may be responsible for minor disagreements in the positions of kidney and liver in the series.

In PNA of various organs the relative incorporations of formic acid-C^{14} bear little relation to its incorporation into the corresponding DNAs. The extents of incorporation of formate into the purines of the PNA of intestine, kidney, and spleen were about 7-, 4-, and 2-fold that into liver, pancreas, and testis (218). But with adenine-N^{15} as the precursor, the sequence liver, kidney, intestine, spleen, testis has been obtained (252). The most striking difference between the two precursors is observed in the liver and intestine; the ratio of incorporation of formate into the liver and intestinal PNA adenines (L:I ratio) was 0.16 (218) and 0.14 (249), that of adenine was 1.7 (cf. 218), and a simultaneous administration of the two precursors confirmed the individual experiments with an L:I ratio from formate-C^{14} of 0.25 and from adenine-N^{15} of 1.6 (133). The results of Abrams (195) with glycine and adenine also show a proportionately better utilization of glycine by intestine, and a sparing by adenine of glycine utilization to a greater extent in the liver than in the intestine. In those experiments the absolute L:I ratio for adenine was 0.84, but might have been influenced by the dose of adenine used, which was 9.5 times that used above.

From the differences between the two types of precursors it was suggested several times (195, 218, 252, 253) that two mechanisms for the synthesis of PNA must exist, and that the purine-utilizing system is relatively more efficient in liver [Drochmans *et al.* (218)] and less so in intestine (195). Orotic acid is also preferentially incorporated into liver PNA pyrimidines (230, 254), and its incorporation does not fully parallel that of P^{32} into the same nucleotides (254). It is obvious that no one precursor gives a reliable picture of the true relative "turnovers" of the various nucleic acid fractions or moieties.

Apparently conflicting evidence accumulated regarding the relative renewals of the PNA and the DNA purines of liver, where the ratio of the incorporation of glycine ranged between 2:1 and 5:1 (71, 195, 222, 255) and that from formate was of the same order (133, 249), while that from adenine was greater than 50:1 (195, 252, 256). Simultaneous administration of adenine-8-C^{14} and glycine-N^{15} (252) and of adenine-1,3-N^{15} and formate-C^{14} (133) confirmed these differences between the utilization of the small precursors and the intact purine in liver. In replicate experiments, in which unfortunately only the total nucleic acid fractions were measured, the renewals of rat liver cytoplasmic PNA and the DNA were in similar low ratios after glycine or formate, and similar high ratios after adenine or P^{32} (257).

The preferential incorporation of adenine into liver PNA exaggerates the PNA:DNA ratio obtained in normal liver, and the general similarity of the results obtained with adenine and P^{32} remains unexplained. The greatly increased incorporation of adenine into the DNA of regenerating liver [Furst *et al.* (252)], or liver tumors (256) seems related to the increased

synthesis of DNA associated with mitosis, and the prolonged retention of adenine (252) or of formate (249) once so incorporated into liver DNA confirms a "biochemical stability" of much of the DNA arising at that time.

Little difference was found between the retention of the isotope of glycine once it is incorporated into liver PNA and DNA purines [Tyner, Heidelberger & LePage (71)], but with formate in normal liver there was a slower loss of isotope from the DNA, and with regenerating liver a still greater difference between the PNA and DNA (249). The glycine experiments (71) were on tumor-bearing animals, and it has now been demonstrated (17, 57 to 62) that the presence of a tumor stimulates DNA metabolism in the liver of the host. Thus an as yet unassessed influence was introduced in those experiments with glycine.

That there is some continuous renewal of DNA of the whole viscera is also shown by the rather large conversion of both pyrimidine nucleosides and nucleotides into the pyrimidines of DNA; while the purine nucleotides led to PNA:DNA ratios of 8 or 10:1, the pyrimidine derivatives led to about 2:1 ratios (219, 238, 239). Interestingly enough a 60:1 ratio, analogous to those found with adenine, was obtained for the incorporation of orotic acid into PNA and DNA pyrimidines of livers of rats (tumor-bearing) [Hurlbert & Potter (230)]. The DNA from a number of organs has been separated into two fractions by centrifugation in isotonic saline, and these can be metabolically differentiated by the extent to which formate is incorporated into their purines and thymine (249). No regular relationship has been found between the individual fractions, or the bases therefrom, in various organs.

In intestine, with all precursors, similar incorporations (133, 195, 249) and similar retentions (249) of the isotopes in both PNA and DNA are found, but here the rapid formation and loss of mucosal cells greatly influence the "apparent metabolic" results obtained, which are the resultant of both metabolism and actual physical loss of cells containing labeled nucleic acid.

The early and rapid renewal of nuclear PNA by a number of precursors continues to be stressed, and at present it remains a potential precursor of cytoplasmic PNAs. However the fact that nuclear PNA is rapidly renewed by adenine (211, 256) and orotic acid (230) under conditions where DNA is not being extensively renewed by those precursors does not particularly favor its candidacy as a precursor of DNA. In the case of cytoplasmic PNA purines, Hammarsten (63) records an impressively complex curve with multiple maxima for the extents of incorporation of glycine at various stages in the regeneration of rat liver. Such a curve could represent a summation of a number of individual components with maxima at different times. A comparison of the incorporation of glycine-N^{15} into various nuclear components showed that while there was little into the DNA there was considerable into the histone and much more into the residual protein (258).

Of necessity, studies directed to the nucleic acids themselves are now be-

ing made on "fractions" which are inadequately characterized mixtures and interpretations of the results are correspondingly difficult. In view of the heterogeneity of DNA as well as of PNA, and of the unequal renewal of individual nucleotides by different precursors, it is obvious that experiments involving measurement of isotope incorporation into gross nucleic acid fractions are becoming of more and more limited value. Attention must turn to characterization of and metabolic studies on more detailed sub-fractions of the nucleic acids (or nucleoproteins).

CATABOLISM

Nucleodepolymerases.—Further evidence has appeared on differences between ribo- and desoxyribonucleases and their inhibitors from diverse sources. Seibert, Lang & Corbert (259) showed that the pH optima varied for the desoxyribonucleases of different organs. Brown, Jacobs & Laskowski (260) confirmed the findings of Maver & Greco (261) concerning the non-identity of the desoxyribonculeases of calf thymus (pH optimum 5, Mg inhibits) and calf pancreas (pH optimum 7, Mg activates), and that the pH optima for the ribonucleases differed (4.5 and 7.5, respectively).[7] Allfrey & Mirsky (262) found desoxyribonuclease activities with pH optima near 5.2 in many tissues including pancreas; they consider these to be the cellular enzymes and the Kunitz enzyme to be secreted by the pancreas as a digestive enzyme.

In agreement with earlier reports, a large portion of each nuclease was reported to be tightly bound in the nuclei, but the highest concentration of ribonuclease was in the particulate fraction (260). Schneider & Hogeboom (265) find the major portion of mouse liver ribo- and desoxyribonucleases to be associated with the mitochondrial fraction. They also find that these enzymatic activities, when liberated by sonic disruption of mitochondria, can be adsorbed by the various particulate fractions and suggest that findings regarding intracellular distributions are complicated by such behavior. The presence of desoxyribonuclease in the cytoplasm (265, 262) is of interest.

The inhibitor of streptococal desoxyribonuclease appears to be a PNA (destroyed by pancreatic ribonuclease), but only bacterial PNAs are effective inhibitors (266). This inhibitor does not show cross inhibition with the desoxyribonuclease inhibitor of yeast (267) which in turn does not inhibit Kunitz's pancreatic ribonuclease. On the other hand, the desoxyribonuclease inhibitors from pigeon crop gland (268), and some rat and human tissues (269, 270) do inhibit pancreatic desoxyribonuclease and form a reversible combination with it [Laskowski *et al.* (269)].

The variations in desoxyribonuclease and inhibitor content of different types of preparations from the same tissue (269) and the growing knowledge

[7] Crystalline beef pancreas ribonuclease has been separated into two active components (263, 264), and there was no evidence for the presence of those two fractions in other tissues (263).

of the tissue specificities and properties (260, 262, 271, 272, 275) of these agents render obsolete surveys of the distribution of nucleases (273, 274),[8] or of inhibitors (270), which involve the use of but one set of conditions, and suggest that future studies must involve extensive characterizations of the enzymes and inhibitors of each tissue studied. Similarly, isolation procedures for DNA which are satisfactory for one tissue may not be so for others, and, as Laskowski *et al.* (260) and Webb (275) have pointed out, the recorded explanations for the success of some procedures (276, 277) now seem incorrect.

The immunologically active type *b* specific substance of *Hemophilus influenzae*, has been characterized as a polyribose phosphate (278) which is susceptible to ribonuclease (278, 279), and this together with the fact that deaminated PNA is also susceptible to ribonuclease (280), emphasizes the caution which must be used in interpreting either structural or histochemical studies with enzymes of incompletely understood specificity.

Nucleosidases.—These enzymes are discussed in more detail by Hestrin (p. 95), but it might be mentioned here that, in addition to the classic phosphorylases, both hydrolases and transglycosidases have been demonstrated. Desoxyribonucleoside phosphorylases show [Manson & Lampen (149)] a considerable similarity to the specificity and reversibility of the ribonucleoside phosphorylase of Kalckar. Hydrolases from *L. pentosus* specific for either purine or pyrimidine nucleosides have been differentiated [Lampen & Wang (281)]. Both a hydrolase and now a phosphorylase (282) have been demonstrated for uridine. In yeast autolysates both a purine nucleoside phosphorylase and a hydrolase were found [Heppel & Hilmoe (283)]. The latter is highly inspecific, cleaving the ribosides (but not other glycosides) of many unnatural purines. One of the outstanding developments is the unequivocal demonstration of true transglycosidation of desoxyribose from one purine or pyrimidine to another by an enzyme from *L. helveticus* [MacNutt *et al.* (167, 284)].

Xanthine oxidase.—The remarkably specific response of the levels of this enzyme in liver to the quantity and quality (285) of the protein of the diet, and the fact that xanthine catabolism by the animals is not altered by low enzyme levels (286, 287) are another fascinating relationship between protein and nucleic acid metabolism. A critique of the methods for determination of the enzyme has appeared (288). The tissue levels are also affected by an unknown dietary factor and the work of Westerfield, Richert, and coworkers has shown that, since intestinal xanthine oxidase is more dependent on this factor than is liver xanthine oxidase, the former may be used as an assay for the factor (289). The dietary factor in milk was concentrated with the xanthine oxidase, and it was suggested that the role of the enzyme in milk might

[8] The method involving measurement of dialyzable P^{32} liberated proposed for ribonuclease assay [Roth (274)] is one heavily dependent upon absolute uniformity of the labeled PNA preparations made available.

merely be to transport this unknown factor to the offspring. The xanthine "oxidase" of chicken liver is of a dehydrogenase character, and a manometric measurement of the production of uric acid subsequently may be made if a hydrogen acceptor (methylene blue) is used under anaerobic conditions (290, 291). The difference in the character of the bulk of the enzyme oxidizing xanthine in mammalian and avian classes may be a reflection of the basic differences in uric acid production in the two.

Xanthine oxidase has been further characterized as to its turnover number, its flavoprotein nature, and the ability of cytochrome-*c* to act as a hydrogen acceptor from it (292). It has been localized in the supernatant fraction of the cell (293). In one of the few demonstrations of the production of an active enzyme *in vitro*, Dhungat & Sreenivasan (294) have shown that liver slices from protein deficient rats (but not from normal rats) are able to synthesize xanthine oxidase when incubated in horse serum, particularly with certain added amino acids. To the numerous compounds which will inhibit the enzyme *in vitro*, flavinoids (295) and ascorbic acid (296) must be added; the latter is the most physiological of the compounds thus effective. The administration of 6-formylpteridin did not affect the xanthine oxidase content of livers (297), although it is a potent inhibitor *in vitro* and was once considered as a possible contaminant responsible for the *in vivo* inhibitory effect of folic acid. Fatterpaker & Sreenivasan report that they find very high levels of folic acid are required to depress the enzyme level (298), and Remy & Westerfield have suggested that it may be that a folic acid deficiency produces an abnormally high xanthine oxidase level, rather than that dietary folic acid depresses it (291). The character of the impurities present in the various samples of folic acid might be partially responsible for the diversity among the reported results.

The action of xanthine oxidase on adenine to form 2,8-dihydroxyadenine has been studied in detail and made the basis of an assay for adenine [Klenow (299)]. Details of the nephrotoxic action of 2,8-dihydroxyadenine deposition in the kidneys have been described (300). The enzyme will also oxidize 2-azaadenine and 2-azahypoxanthine (301).

The catabolism of purines.—A paper chromatographic method for microestimation of enzyme actions, some studies of deamination of adenine by *E. coli* and of cytosine (and 5-methylcytosine) by *E. coli* and yeast, and the fact that extracts of rabbit liver powder will deaminate guanine, 8-azaguanine, and adenosine, but fail to deaminate adenine, 2,6-diaminopurine, cytosine, and cytidine are reported by Kream & Chargaff (302). *E. coli B* will deaminate isoguanine and 2,6-diaminopurine as well as other purines [Friedman & Gots (303)].

The general trend of results with isotopically labeled purines has confirmed that allantoin is the major end product of purine metabolism in lower mammals. The findings that negligible amounts of C^{14} reached carbon dioxide or urea from adenine-8-C^{14} (205), guanine-8-C^{14} (194), or 2,6-diaminopurine-2-C^{14} (306), but that considerable amounts did so from adenine-4,6-C^{14} (pre-

sumably from the 6-C) (211), are in accord with this. The former prove that not more than minute traces of purines are completely degraded. No degradation of uric acid to alloxan by liver homogenates (308) nor *in vivo* (309) was found, and it is felt that there is no support for the postulate (310) of a conversion of uric acid to alloxan in uric acid diabetes. Rat liver uricase has been located in the large granules (311).

In man intravenously injected uric acid-1,3-N^{15} was chiefly recovered as such in the urine (312, 313, 314), although orally administered uric acid was extensively degraded to urea, and only 9 per cent was recovered unchanged (313). Benedict *et al.* (312) had observed small amounts of N^{15} in urea after intravenously administered uric acid, and Buzard, Bishop & Talbot (315) have now found that the extent of such degradation varies with the individual; in six to nine days the recoveries as uric acid were from 68 to 77 per cent in five individuals and 94 per cent in one. The extensive degradation of orally administered uric acid can probably be attributed (313) to bacterial action in the intestine; the excretion of some of the intravenously administered uric acid into the intestine via the bile (316) and reabsorption of bacterial degradation products may explain its variable catabolism without requiring a splitting of uric acid by mammalian tissues. Griffiths (317) has extended his observation (318) that uric acid may be oxidized by cytochrome-*c* at pH 7.9 (and also by alkaline copper), but the product, "dehydrouric acid," retains the intact purine ring, and possible significance in organisms lacking uricase remains obscure.

The metabolism of uric acid in gout is under study in the laboratories of Stettin, Benedict & Gutman, and of Talbott & Bishop. With intravenously administered uric acid in the normal man, there is a miscible pool of about a gram (312, 313, 314, 319) in a volume approximately corresponding to the extra cellular space (313). In the initial studies where the first urine collection periods were six or twelve hours immediate equilibration with the injected uric acid seemed to occur, but later (320) it was shown that the equilibration was not complete for several hours. In the gouty patient (312, 314, 319, 321) a much larger "miscible pool" may be found, and equilibration with the bulk of the tophi is not facile. With glycine-N^{15} in a gouty individual a more rapid incorporation into, and loss from, the uric acid was found (322), and from this it was concluded that an abnormally large production of uric acid *de novo* (reminiscent of that occurring in avian and reptilian species) may be characteristic of gout.

Experiments involving uric acid-N^{15} also reaffirmed that uric acid excretion is increased by high purine or high protein diets and is not affected by carbohydrate or exercise (323). Plasma uric acid levels do not vary greatly with age (324).

There is a renewed interest (325, 326) in uric acid riboside, and Carter & Potter (326) report that a preparation from erythrocytes is not spectrally identical with that reported by Falconer & Gulland (327). Comparison with that reported by Davis, Newton & Benedict (328) was not possible.

The fermentation of purines by new strains of anaerobic bacteria has been studied. As with *Clostridium acidici-urici* (329), the reactions are complex, and multiple pathways seem to be involved. *Micrococcus lactylicus* 416 brings about a propionic acid fermentation of hypoxanthine, xanthine, or uric acid, while *M. lactylicus* 221 degrades the purine ring less readily but will convert hypoxanthine into xanthine plus hydrogen (330). With strain 416 an atmosphere of hydrogen partially blocks the decomposition and a similar conversion of hypoxanthine to xanthine is noted. In nitrogen, xanthine undergoes dismutation into uric acid and the degradation products of hypoxanthine. *M. aerogenes* will ferment all the common purines and the pyrimidines to a smaller extent, and chromatographic evidence indicated the production of thymine and uracil during fermentation of adenine or hypoxanthine, but of uracil only from guanine or xanthine (331). The utilizations of the nitrogen of a number of purines (332) and pyrimidines (333) by *T. utilis* and *S. cerivisiae* have been compared, the former yeast completely degrading many while the latter will apparently utilize only the substituent amino groups. Less efficient degradation of the nucleotides and none of intact PNA or DNA was found, although both yeasts will fully degrade allantoin. There was no evidence of any degradation of isotopically labeled purines by *E. coli* (200) or *L. casei* (202). The first report of the presence of allantoicase in insects (334) and a review of this enzyme (335) have appeared.

In the catabolism of caffeine the detection of some of 7-methyl and 1,7-dimethyl uric acids are reported (336), though the chief product is 1-methyl uric acid (336, 337). From theophylline the primary products are 1-methyl (337) and 1,3-dimethyl (337, 338) uric acids, and it appears that it is the 3 position which is most readily demethylated.

Catabolism of pyrimidines.—In the intact mammal a number of examples of interconversion of derivatives of uracil into those of cytosine, and vice versa, are known. There are new examples of deamination of cytosine to uracil by bacteria (302), and recently Gunzalus & Tonzetich (339) have shown that transamination from cytosine (or from amino purines or pyridoxamine) to α-ketoglutarate is catalyzed by a preparation from *E. coli.*

Soil bacteria (340 to 344) and yeasts (333) utilize a number of pyrimidines as sources of carbon dioxide and ammonia. Barbituric acid (341, 343) and 5-methylbarbituric acid (343, 344) have been identified as intermediates in oxidations of uracil and thymine by soil bacteria, and in some instances thymine may yield barbituric acid, possibly via demethylation to uracil (342). However, from utilization studies (333), it appears that yeasts degrade uracil via hydrouracil and hydroorotic acid. It has also been claimed (345), on the basis of spectral evidence, that a phosphate derivative of 5-hydroxymethyluracil is a product of bacterial oxidation of thymine. Speculations on pyrimidine catabolism in the mammal have not involved barbituric acid as an intermediate, but it has recently been claimed, also upon spectral evidence, that barbituric acid arises from uracil by the action of rat liver preparations (346). However the β-amino-isobutyric acid in human and

animal urines, which arises from thymine (347), must arise via a pathway not involving a barbituric acid.

Catabolism of the anesthetic barbiturates was formerly thought to involve extensive degradation of the ring, but experiments with pentobarbital-1,3-N^{15} (348, 349) and 2-C^{14} (350) indicated that little of the N^{15} and no C^{14} reached urea. It now appears that a major fate of several 5,5-dialkylbarbiturates is the introduction of a hydroxyl at the γ-carbon of the longer 5-alkyl group (351), but that with analogous 2-thiobarbiturates the oxidation of an ω-carbon to a carboxyl is involved (352, 353).

Miscellaneous

Transforming principles.—These have been reviewed by Austrian (354) and Hotchkiss (355). The latter has summarized some 40 known transforming factors, their relationships, and their character. That of *H. influenzae* (356) has been used by Zamenhof (357), to demonstrate additional parallelisms of DNA-like properties and the transforming activity, and to confirm that minor alterations in the DNA molecule may result in loss of the biological activity. Ephrussi-Taylor (305) and Austrian (358) discuss the possibility that they may replace, or displace, portions of the nucleic acid of the cells which are to undergo transformation.

Virus composition.—Knight (359) applied the Markham & Smith methods (360) to the analysis of the base composition (in some cases to both the PNA and to the intact ribonucleoprotein) of a number of tobacco mosaic and cucumber viruses and found a remarkable uniformity of composition of six strains of tobacco mosaic virus, and agreement with the values for three out of five tobacco mosaic virus strains reported and one cucumber virus reported by Markham & Smith (361). Knight (359) tentatively proposes that the mutant strains vary in the amino acid composition of their proteins [but some differences must be less than the precision of amino acid analyses thus far applied (362)].

Reproduction of virus nucleic acid.—In most instances the majority of the P, N, and desoxyribose of viral (bacteriophage) nucleic acid is derived from the medium after infection of the bacterium, although from 15 to 40 per cent of the virus DNA is derived from bacterial DNA present at the time of infection (363 to 366). However, the T_7 coliphage derives nearly all its DNA from the host, presumably since there is sufficient host DNA to supply that needed for the progeny of this small phage [Putnam *et al.* (367)]. In addition, direct evidence has now been obtained that the infecting particles are broken down and partially re-utilized and that it is the nucleic acid portion which is degraded more extensively in the early stages. The extent of breakdown was reported [Kozloff (368, 369, 370)] to be independent of the number of infecting particles, in contrast to the report of Lesley *et al.* (371) that the phage particles first absorbed are broken down far less extensively than those absorbed later.

By the use of *E. coli* in which the pyrimidines had been labeled by ad-

ministration of orotic acid-C^{14}, Weed & Cohen (225) have demonstrated that pyrimidines of the host can also be utilized by the phage. The more extensive labeling of the virus pyrimidines found when early lysis was artificially induced indicated that host compounds are utilized more extensively in the earlier stages of viral synthesis.

With bacteria in which the purines were previously labeled, and with labeled purines in the medium, Koch, Putnam & Evans have shown (200) that host purines which are re-utilized are transferred intact with respect to both the ring carbons and amino groups. There was no evidence of degradation of the purines or of their synthesis from nonpurine bacterial N. The percentage of the phage DNA guanine derived from the host is greater than that of the adenine, and this could be correlated with the fact that the host DNA contains relatively more guanine than adenine and the phage DNA more adenine than guanine, although the possibility does not seem to be excluded that the results might be explained by a preferential utilization of adenine arising *de novo* for the synthesis of phage. Either labeled adenine or guanine, when added to the medium after inoculation, could be used for phage synthesis, but the different ratios of isotope incorporation which were obtained showed that purine interconversion, more particularly the conversion of guanine into adenine, was significantly decreased in the infected cell.

The extent to which virus bases were derived from PNA or DNA bases could not be distinguished in these studies, but from the fact that the purines (200), like phosphorus (363), are not renewed in the PNA of the infected cell, it is presumed that host DNA is the major source. The only information as to the size of the molecules transferred is that regarding the integrity of the purines; other conclusions are in the realm of speculation at present.

The old conclusion that the viral replication involves a distortion of the normal metabolic characteristics of the host receives further documentation from other observations. In *Bacterium coli* infected with phage T_5 (a DNP), a uracil synthesis soon ceases while thymine synthesis is accelerated (372); in *E. coli* [Friedman & Gots (373)] and in mammals [Bauer (374)] the presence of a virus alters the host purine and nucleoside enzyme patterns; in mouse brain it alters the amino acid synthesis pattern (375).

Metabolism of some inhibitors.—The mass of antimetabolite and chemotherapeutic trials with nucleic acid derivatives have led to many biological effects and to many suggestions as to metabolic pathways. Only a few studies which reveal concrete metabolic fates of compounds with demonstrated inhibitory actions are mentioned here. In the monkey and mouse, 8-azaguanine-4-C^{14} or 2-C^{14} was found to be almost quantitatively excreted in the urine as azaxanthine *in vivo* (376), and it was demonstrated that there is less azaguanine deaminase in certain susceptible tumors than in nonsusceptible tumors and normal tissues (25). Traces of azaguanine-2-C^{14} were found to be incorporated into the nucleic acids of the mouse (377), and with *T. geleii* grown in its presence it could be found in the nucleic acid fractions (378). The thymine antagonist 5-bromouracil-Br^{82} was also found to be extensively

incorporated into the nucleic acid fraction of *Streptococcus faecalis* (379). 2,6-Diaminopurine is transformed into PNA adenine and guanine in *L. casei*, and the only influence of inhibitory levels was to reduce the interconversion of the purines to a small extent (202). No evidence was found of a direct incorporation of diaminopurine into the nucleic acids in *L. casei*, but minute amounts of it are incorporated into nucleic acids in the mouse (306). It is interesting that such compounds seem to be "bound" in the nucleic acid fractions, but as yet no isolation of a product larger than the compound administered, e.g., a nucleoside, has been accomplished and such evidence is desirable. 2,6-Diamino-9-β-D-ribofuranosylpurine is the only compound other than adenosine found to be deaminated by adenosine deaminase (380) or phosphorylated by adenosinekinase (237), and it has been proposed (202) that it is some metabolic product of diaminopurine which can compete with a corresponding metabolic product of adenine.

Compounds related to nucleic acids.—Several newly detected or identified compounds must be kept in mind as candidates for positions in metabolic schemes. Penicillin has been observed to interfere with the catabolism of pentose (381), of ribonucleosides, and nucleotides (382), and to cause the accumulation of acid-labile phosphate (383) and free nucleotide (384) fractions. Park has isolated (385) and characterized (386) three compounds accumulated by *S. aureus* during the first 30 minutes after the addition of penicillin. Uridine-5′-pyrophosphate linked to an N-acetyl amino sugar acid is common to all. In addition, one contains alanine and another a peptid of D-glutamic acid, L-lysine, and three alanine residues. Park has speculated (387) on the possibility that the large amount of uridine thus isolated might have been diverted from normal nucleic acid synthesis or that such nucleotide-amino acid derivatives could function for amino acid transfer. The type of linkage present in these compounds could also offer a possible explanation for the stability of some PNA-protein linkages.

The appearance of uridine diphosphate in the above compounds as well as in uridine diphospho-glucose (UDPG) [Caputto *et al.* (388)] is noteworthy. The function of UDPG as a coenzyme in the conversion of galactose-1-phosphate to glucose-1-phosphate has been demonstrated (389), and Leloir (390) has also demonstrated an enzymatic conversion of UDPG to uridine diphospho-galactose, presumably by an epimerization at the C-4 such as occurs *in vivo* in the conversion of galactose to glucose (391). Further studies of the character of UDPG have shown that alkaline degradation leads to a 1,2-cyclic phosphate of glucose, that a component UDPX (not uridine diphospho-galactose, nor the *S. aureus* compounds) is also detectable (392), that it may arise from uridine diphosphate, ATP, and glucose-1-phosphate [Trucco (393)], and that pyrophosphorolysis leads to uridine triphosphate [Kalckar & Cutolo (394)]. Buell also reports (395) that uridylic acid is present in the nonprotein nitrogen fraction of rabbit muscle phosphorylase. Leloir has also mentioned (396) the detection of a nucleotide containing guanine, mannose, and phosphate. A riboside of orotic acid (oroti-

dine) has been obtained from a *Neurospora crassa* mutant (227); spongothymidine (397) has been characterized as a xylofuranoside (398), and a reinvestigation of the structure of vicine has resulted in revision of its structure to 2,4-diamino-5,6-dihydroxypyrimidine with glucopyranose apparently attached to the oxygen of the 5 position (399). A hydrouracil from beef spleen is reported (307).

Adenine has also appeared in previously unidentified roles. Adenine thiomethylriboside [now fully identified as 9-β-D(5′-desoxy-5′-methylthiol) ribofuranosyl adenine (400 to 403)] has been shown to be accumulated as a "reservoir" of sulfur and methyl groups by yeast grown with sufficient methionine [Smith & Schlenk (404)]. An intermediate formed in the "activation" of methionine by ATP and an enzyme from liver has been reported by Cantoni (189, 304) to be a sulfonium compound composed of the 5′-desoxyadenosine moiety attached to the sulfur of methionine. This interesting compound possesses the full skeletons of both adenine thiomethylriboside and methionine and represents an entirely new type of coenzyme in which adenine participates. In the phosphorylated form of coenzyme A (405) it is the *b* position of the adenosine which proves to carry the additional phosphate (406) in contrast to triphosphopyridine nucleotide where it is the *a* position of the adenosine which carries the third phosphate (407). In pseudovitamin B_{12}, which serves as a growth factor for some microorganisms, adenine is found in lieu of 5,6-dimethylbenzimidazole [Dion, Calkins & Pfiffner (408)]. The possibility is intriguing that this adenine might be in an α-glycosidic linkage, as is the benzimidazole of B_{12}. In this connection the ability of adenine to complex with cobalt (409) might be noted. Cordycepin, a nucleoside produced by *Cordyceps militaris* and possessing limited antibiotic activity, has been identified as 9-(3′-desoxyapiofuranosyl)-adenine (apiose = $CHO \cdot CHOH \cdot COH(CH_2OH)_2$) (410).

Symposia and Reviews

An able review of purine and pyrimidine metabolism by Christman (411) covers the period since the review by Rose in 1923. Needham has reviewed the role of ATP in the proteins of muscle (412), and Mirsky the composition of the nucleus (42). Collected papers from Symposia on Biochemistry of Nucleic Acids, Oak Ridge, 1950 (413), Chemistry and Physiology of the Nucleus, Brookhaven, 1951 (414), Genes and Mutations, Cold Spring Harbor, 1951 (415) have appeared.

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CARBOHYDRATE METABOLISM[1,2]

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Introduction

Work during 1952 has been concentrated mainly on the same lines as in the previous year. The reactions involving coenzyme A have been the center of attraction and the mechanism by which ATP[2] and acetate yield acetyl CoA[2] has been elucidated.

Knowledge of the oxidation of α-keto acids and the "pyruvate oxidation factor" has advanced considerably. The transformations of pentose phosphates to give a two and a three-carbon atom intermediate are becoming better known. Work on the action of insulin continues to indicate that the first step in glucose utilization is affected as well as some reaction at the two-carbon stage.

New fields in which interest is increasing include the metabolism of hexosamines and uronic acids. The reviewers regret that for reasons of space many interesting papers have had to be omitted.

Metabolism of Poly- and Disaccharides

Amylolysis.—Peat *et al.* (1) described a new enzyme: the Z enzyme, which is necessary for the complete hydrolysis of amylose. Pure preparations of β-amylase hydrolyze only about 70 per cent of the amylose, whereas if the Z enzyme is present, hydrolysis reaches completion. The Z enzyme appears to hydrolyze a limited number of branch links in amylose which act as barriers to β-amylolysis or phosphorolysis. Evidence was presented indicating that these links have a β configuration and that the Z enzyme acts as a β-glucosidase.

It has been assumed that α-amylase acts on amylose by the random scission of all except the terminal α-1,4 linkages. Thus, the products should be maltose and maltotriose, which only contain terminal linkages. A statistical treatment predicts that the ratio of the two substances should be 2.35 to 1 in the final mixture. This hypothesis has been examined by Roberts & Whelan (2). They found the ratio of maltose:maltotriose to be 2.39:1. Moreover, maltotriose was not hydrolyzed by salivary amylase, and higher oligosaccharides of 4 to 7 glucose units yielded the predicted amounts of maltose and maltotriose, thus confirming the theory.

[1] This review covers the period from January to September, 1952.

[2] The following abbreviations are used: AMP for adenosinemonophosphate; ATP for adenosinetriphosphate; DPN for diphosphopyridine nucleotide; $DPNH_2$ for diphosphopyridine nucleotide (reduced form); CoA for coenzyme A; TPN for triphosphopyridine nucleotide; $TPNH_2$ for triphosphopyridine nucleotide (reduced form); UDPG for uridine diphosphate glucose.

Branching enzymes.—A method for the purification and crystallization of the Q enzyme, which forms amylopectin from amylose, was described by Gilbert & Patrick (3). The enzyme was found to be activated by ammonium sulphate. It was obtained free from phosphorylase and still gave rise to reducing groups from amylose, although to a smaller extent than the crude enzyme.

The minimum length of the amylose chains required for activity has been determined. About forty glucose residues are needed. Thus, Bailey *et al.* (4) found a very slow action on a polysaccharide of average chain length 30 and a twentyfold increase for length 58. Similar results were obtained by Nussenbaum & Hassid (5), who also produced evidence showing that the Q enzyme transfers degraded amylose fragments to a number of other amylose molecules forming branched structures. Sri Ram & Giri (6) have isolated from green gram (*Phaseolus radiatus*) a Q enzyme with similar properties to those of the potato enzyme. The Q enzyme of a protozoan, *Polytomella coeca*, was isolated by Bebbington *et al.* (7, 8). The synthesis of starch in this organism takes place by the joint action of phosphorylase and Q enzyme. A glycogen-amylopectin type of polysaccharide could be synthesized with potato phosphorylase and *Polytomella* Q enzyme.

Phosphorylases.—Fitting & Scherp (9) found a strain of *Neisseria* which oxidizes maltose much faster than glucose; this is another case of "direct oxidation." Further studies with Doudoroff (10) led to the extraordinary finding that the extracts catalyze the reaction:

$$\text{Maltose} + \text{inorganic phosphate} \rightleftarrows \text{glucose} + \beta\text{-glucose-1-phosphate} \qquad 1.$$

Evidence was obtained for the reversibility of the reaction. This is the first time that β-glucose-1-phosphate, which had been suggested as a precursor of cellulose, has been obtained by enzyme action.

The action pattern of potato polysaccharide phosphorylase was investigated by Bailey & Whelan (11), who used maltohexose as primer and examined the products. It was concluded that all the chains grow gradually and that this result excluded the single chain hypothesis (successive synthesis of high weight molecules).

Trevelyan *et al.* (12) carefully redetermined the equilibrium constant of the polysaccharide phosphorylase reaction. At 30° the free energy change for the reaction:

Acid glucose-1-phosphate→acid orthophosphate+polysaccharide was calculated as −1460 cal. per mole. The effect of magnesium ions on the equilibrium was also considered from the theoretical and experimental point of view, and it was concluded that little effect due to Mg^{++} on the equilibrium is to be expected under physiological conditions. A new method of purification of potato phosphorylase (13) has been described, as well as the isolation of a starch phosphorylase from green gram (*Phaseolus radiatus*) (6).

Transglycosidation.—Active work has continued on transglycosidation reactions and new cases have been discovered. However, quantitative data

on the relation between rate transglucosidation to water and to other acceptors are still lacking and nothing is known on the physiological significance of many of these transfer reactions.

An enzyme preparation from *Lactobacillus helveticus* was found to catalyze the transfer of the desoxyribosyl group from one purine or pyrimidine to another (14). Many desoxyribosides were prepared in this manner including some previously unknown, such as that of uracil. Under the conditions of the experiments about 80 per cent of the desoxyriboside groups were transferred and only about 20 per cent hydrolyzed. The intermediate formation of phosphorylated compounds was excluded. Moreover, Kalckar *et al.* (15) used labeled adenine in order to exclude other conceivable mechanisms such as transamination. They found that C^{14} adenine desoxyribose was formed from hypoxanthine desoxyriboside and C^{14} adenine with no decrease in the concentration of label.

Another interesting reaction was discovered by Whitby (16). A liver enzyme can transfer a glucosyl residue from maltose or glycogen to riboflavin. The glucopyranosyl group becomes fixed to the 5′ position of the ribityl moiety, probably in the α configuration. Glucose and glucose phosphate behave as inhibitors.

Further study of the products formed in the sucrose-invertase reaction revealed the presence of oligosaccharides with a fructose/glucose ratio of 1, 2, and 3 (17). When the invertase-sucrose reaction was carried out in the presence of alcohols such as methanol, ethanol, n-propanol, or benzyl alcohol, it was found that the corresponding fructosides were formed (18).

Experiments with an invertase preparation from honey, which acts as glucoinvertase in contrast to that from yeast, which is a fructoinvertase, led to the detection of six oligosaccharides. They appeared to be different from the compounds formed by yeast invertase, as would be expected since a transglucosidation and not a transfructosidation would be involved (19). A fructosyl transferring enzyme of *Aspergillus orizae* was studied by Pazur (20). With sucrose as fructosyl donor and acceptor, a trisaccharide with fructose/glucose ratio of 2 was formed, which in turn could serve as acceptor, giving a tetrasaccharide of ratio 3. Similar events occurred using raffinose.

With an extract of Jerusalem artichoke tuber, Dedonder (21) found that sucrose was transformed into oligosaccharides of fructose/glucose ratio of 2, 3 or more. Inulin as well as sucrose could serve as fructosyl donor. These same substances could also be found preformed in the tuber. With extracts prepared from the stems, phosphate was reported to be required for oligosaccharide formation, but no evidence of the formation of phosphorylated intermediates was obtained.

Transglucosidation from maltose was found by Pazur & French (22) to be catalyzed by *Aspergillus orizae* extracts. All the products contained 1,6 linkages. The results were interpreted as follows: in the first stage a glucosyl enzyme complex is formed from maltose. The energy state of this complex is such that the free energy change for the reaction in the direction

of synthesis of 1,6 linkages is negative, while the free energy change for the reaction in the direction of synthesis of 1,4 linkages is positive. Experiments with radioactive glucose proved that enzyme action involved a transfer of glucosidic units from the 4 to the 6 position of the acceptors. The reaction products were carefully identified as: isomaltose; two trisaccharides; panose (4-isomaltosylglucose) and dextrantriose (6-isomaltosylglucose); and a tetrasaccharide (4-dextrantriosylglucose). It was not stated whether the enzyme is believed to be different from maltase. Synthesis of isomaltose with *A. orizae* enzymes has also been obtained starting with 47 per cent glucose solutions (23).

In contrast with these enzymes which lead to the formation of 1,6 linkages, it is known that the enzyme amylomaltase from *E. coli* leads to the formation of α-1,4 linkages. It catalyzes the reaction: n maltose$\rightleftharpoons$n glucose+(glucose)$_n$. The reaction products were studied by Barker & Bourne (24).

Transgalactosidation during lactose hydrolysis was studied by Aronson (25). Four oligosaccharides were found to be formed: (*a*) an isomer of lactose, (*b*) a digalactoside, (*c*) an oligosaccharide with a galactose/glucose ratio of 2, and (*d*) another compound which was not analyzed. Increasing concentrations of substrate increased the yield of oligosaccharides as would be expected, since there would be a competition between water and other acceptors for the galactosyl residues. Evidence was obtained for a transgalactosidation to glycerol or xylose.

Sucrose.—Meeuse *et al.* (26) found an increase in sucrose after aerobic incubation of potato slices in the presence of glucose or fructose. Inorganic phosphate inhibited this change. Incubation under anaerobic conditions was ineffective unless ATP[2] was added. Many of the glycolytic enzymes were detected as well as a phosphatase acting mainly on glucose-6-phosphate. Some evidence for the presence of saccharose-phosphorylase was also reported. Bauer & Gemmill (27) measured the heat produced in the enzymic hydrolysis of sucrose. Values of 3.28 and 3.37 Kcal./mole were obtained with two methods.

Lactose.—Reithel *et al.* (28) have obtained lactose synthesis on incubation of homogenates of lactating mammary gland of guinea pigs with glucose, glycogen, or glucose-1-phosphate. Lactose synthesis was followed by colorimetric and chromatographic methods and also with C^{14}-glucose, which was found to be incorporated into lactose. Esters such as β-galactose-1-phosphate and glucose-4-phosphate, produced no increase in lactose synthesis. No formation of lactose-1-phosphate could be detected nor was this substance transformed by the homogenates (29).

Caputto & Trucco (30) incubated mammary gland slices with radioactive glucose and separated the products by chromatography. Besides the radioactive glucose and lactose spots, two galactose containing slower running substances were radioactive. One of them has been studied further (31) and found to be a compound of lactose and a nitrogen-containing unidentified substance. The lactose formed in these experiments contained approximately

equal radioactivity in the glucose and galactose portions. Studies with gland extracts revealed the presence of UDPG[2] and also of the enzyme system which transforms galactose-1-phosphate into glucose-6-phosphate.

Malpress & Morrison (32) carried out a chromatographic study of the substances formed on incubation of mammary gland slices with glucose. Several spots were found and one of them was believed to be α-galactose-1-phosphate, which they report as having been identified as a normal trace constituent of milk.

After injection of 1-C^{14}-glucose to lactating goats, Barry (33) isolated the lactose and studied the tracer distribution in the glucose and galactose moieties. It was concluded that the transformation of glucose into galactose is probably a direct process not involving breakdown into smaller fragments. Similar results were obtained by administration of 1-C^{14}-glucose to rabbits (34).

Hexoses and Related Substances

Glycolysis.—Results consistent with the operation of the Embden-Meyerhof scheme have been obtained in: *Paramecium caudatum* (35), *Clostridium butyricum* (36), *Brucella suis* (37), *Chlorella* (38), preosseus cartilage (39), and white blood cells (40).

A mechanism different from both the Embden-Meyerhof scheme and the hexosemonophosphate shunt was described by Entner & Doudoroff (41). With intact cells or crude enzyme preparations of *Pseudomonas saccharophila* it was shown that glucose and gluconic acid could be utilized in such a way as to yield two pyruvic acid molecules, the carboxyl group of one of which was derived from the 1-C of glucose. The initial reaction appeared to be a phosphorylation of glucose to glucose-6-phosphate followed by oxidation to 6-phosphogluconic acid and cleavage to yield pyruvic acid from the first three carbon atoms and triosephosphate from the last three. Glyceraldehyde phosphate was transformed into pyruvic acid when dinitrophenol was used as inhibitor and into methylglyoxal in the presence of iodoacetate.

Another organism in which glucose utilization appears to depart from the Embden-Meyerhof scheme is *Leuconostoc mesenteroides*, which brings about an heterolactic fermentation. Each mole of glucose yields one mole of lactate, ethanol, and CO_2. Aldolase and isomerase do not function in the fermentation, but the enzymes required for lactic acid formation from triose phosphate are present. Experiments with 1-C^{14} and 3-4-C^{14}-glucose carried out by Gunsalus & Gibbs (42) showed that the 1-C of glucose gives rise to CO_2. Lactate seems to arise from carbons 4, 5, and 6, and ethanol from carbons 2 and 3. It was also found that acetate is reduced to ethanol during the fermentation. Glucose utilization in these organisms thus appears to involve a pathway in which glucose would first be oxidized to a 5-carbon substance; this substance would then split into triose and a C_2 compound, which eventually is transformed into ethanol.

Direct oxidation.—From measurements of the glucose-6-phosphate de-

hydrogenase activity of liver homogenate supernatants, Glock & McLean (43) reached the conclusion that this oxidation pathway must be of physiological significance and that it must be in active competition with the glycolytic pathway.

Enzymes from liver and bone marrow oxidizing 6-phosphogluconate were described by Seegmiller & Horecker (44). The reaction products were ribose phosphate, ribulose phosphate, and CO_2. With a crude enzyme preparation from bone marrow using TPN[2] as oxidizing agent, it was observed that at first phosphogluconate disappeared while pentose phosphate increased, and afterwards pentose phosphate also disappeared and glucose-6-phosphate was formed.

The preparation and properties of glucose-6-phosphate and 6-phosphogluconate dehydrogenases of *E. coli* were described (45). An extract was obtained from *Aerobacter aerogenes* (46) oxidizing glucose, glucose-6-phosphate, pyruvate, and succinate. Higher plant enzymes were found to catalyze the over-all oxidation of glucose-6-phosphate to pentose phosphate (47).

Cori & Lipmann (48) showed that glucose-6-phosphate oxidation gives rise to phosphoglucono-lactone as first oxidation product. That is, the pyranose form of glucose-6-phosphate is dehydrogenated to the δ-lactone without disturbing the ring.

Glucose dehydrogenase from ox liver was purified by Strecker & Korkes (49). Either DPN[2] or TPN could be used as oxidants. The reaction is reversible; at pH 6.7 the equilibrium constant was found to be 15, which corresponds to $E_0' = -0.44$ volt. The transformation of glucono-lactone to gluconic acid occurred nonenzymatically. The enzyme acts on β-glucose but not on α-glucose, and slowly on xylose. Such a specificity for the β-anomer is a feature shared with notatin (50). Extracts of *Penicillum notatum* were found to contain an enzyme which catalyzes the reversible transformation of the α- into the β-anomer of glucose (51).

Glucose and derivatives.—Long (52) determined the best conditions for the detection of glucokinases in tissue extracts. Many rat organs were examined, and it was found that the most active was brain and the least active liver. The low activity of liver was not considered surprising since normally liver glycogen would be formed from C_3-fragments produced from glucose in the peripheral tissues, that is, by a mechanism not involving glucokinase.

Yeast hexokinase has been found to phosphorylate 2-desoxy-D-glucose and this sugar was described as an inhibitor of yeast fermentation (53). Yeast gluconokinase, which catalyzes the phosphorylation of gluconate to 6-phosphogluconate was studied by Sable & Guarino (54). The enzyme was found to be different from hexokinase.

Fructose and derivatives.—Additional work was carried out on liver fructokinase. Hers (55) with a twentyfold purified preparation, confirmed that both fructose and sorbose are phosphorylated and found that potassium ions exert an activating effect. The affinity of ATP[2] for the enzyme was five times

greater in the presence of K^+ than with Na^+. The optimum amount of Mg^{++} depended on ATP concentration. With low cation concentration maximal activity was obtained with Mg/ATP = 0.5.

The purification, properties, and method of estimation of the phosphohexokinase of higher plants were described by Axelrod *et al.* (56).

The amount of fructosediphosphate in liver tissue was found to increase about fourfold after incubation at 38° and pH 4.5. The activation was considered to be proteolytic in nature and was also obtained with papain (57).

An interesting finding has been the detection of fructose in rabbit liver glycogen. After hydrolysis with α-amylase, Peat *et al.* (58) were able to detect a disaccharide which was identified as maltulose (4[α-glucopyranoside] fructose) by comparison with a synthetic specimen.

Fructose has been found to stimulate up to 80 per cent the disappearance of ethanol from blood (59). The stimulation obtained with glucose was of only 10 per cent. Ethanol excretion in urine was equal in all cases, so that the fructose effect was attributed to an increased metabolism of ethanol.

Galactose.—The reversibility of the transformation of glucose-1-phosphate into galactose-1-phosphate has been proved by using crude extracts of *Saccharomyces fragilis* in the presence of arsenate, thus eliminating the interference by phosphoglucomutase. Starting with any of the two substances an equilibrium mixture was formed which contained about 25 per cent of the galactose ester and 75 per cent of the glucose ester (60).

The galactokinase of *S. fragilis* has been found to be inhibited by—SH blocking compounds and to be reactivated by glutathione (61). Using partially purified preparations it has been possible to detect galactokinase in liver with certainty (62).

Hydrolysis of glucose-4-phosphate with simultaneous inversion to yield galactose has been conceived as a possible mechanism of glucose-galactose interconversion. However, Dursch & Reithel (63) found that hydrolysis of glucose-4-phosphate with alkaline or acid phosphatase or with acid gives glucose and not galactose.

Studies have been carried out on the action of alkali on the coenzyme of the glucose-1-phosphate→galactose-1-phosphate transformation (UDPG[2]). It was found (64) that the products of alkaline degradation are uridine-5′-phosphate and a glucose monophosphoric ester doubly esterified at positions 1 and 2 of the glucose. Further treatment of the cyclic ester gave about three parts of glucose-2-phosphate and one part of glucose-1-phosphate. *Streptococcus pyogenes* was found to produce two molecules of lactic acid from glucose, while it only produced one from galactose (65).

Mannose.—The conversion of 1-C^{14} mannose into liver glycogen in rats has been studied (66). About 85 per cent of the radioactivity remained in 1-C of the glucose residues, thus indicating that conversion takes place without breakdown of the carbon chain.

Pasteur effect.—Experiments bearing on the mechanism of the Pasteur

effect were carried out by Fowler (67) in *E. coli*. When the cells were transferred from aerobiosis to anaerobiosis they ceased to grow and to use glucose for 30 to 40 minutes. After that both phenomena started abruptly and the glucose uptake became 2.5 times higher than aerobically. On transferring again to aerobiosis the fermenting capacity declined after a short time. Dinitrophenol prevented this decline. It was concluded that the enzyme system for anaerobic utilization is adaptative and that the Pasteur effect would consist in an inhibition by oxygen of the formation of the enzymes of fermentation. In previous theories it was supposed that oxygen inhibited certain enzymes but did not affect their formation.

Formation of glucose from fatty acids.—In order to study the mechanism of incorporation of the carbon from natural fatty acids into glucose, Abraham, Chaikoff & Hassid (68) injected palmitate labelled in 1-C or 6-C to diabetic dogs. In the experiments with palmitate 1-C^{14} all the radioactivity was found in equal amounts in 3-C and 4-C of glucose. With 6-C^{14} palmitate the label was distributed symmetrically in the six carbon atoms of glucose. This pattern of incorporation may be explained by β-oxidation of the palmitate to carboxyl labelled acetyl residues followed by conversion to three-carbon units by way of the tricarboxylic acid cycle and condensation of three-carbon units to glucose, according to the Embden-Meyerhof scheme.

Metabolism of amino sugars.—Chou & Soodak (69) found an enzyme in pigeon liver which transfers acetyl from acetyl CoA[2] to glucosamine, and more slowly to galactosamine. It seems to be different from the enzyme which acetylates arylamines.

The formation of hyaluronic acid in group A hemolytic streptococci from glucose 1-C^{14} was studied by Topper & Lipton (70). Equal amounts of C^{14} were found in the two components of hyaluronate: glucosamine and glucuronate. The 1-C of glucosamine contained about five times as much label as the rest of the molecule, thus indicating a direct conversion of glucose into glucosamine. A comparison of hyaluronate formation from glucose and galactose in *S. pyogenes* showed that only one-third to one-half as much was formed from the latter (65).

Phosphorylation of galactosamine by ATP[2] has been found to be catalyzed by an enzyme from liver and also by extracts of a lactose fermenting yeast (62). In both cases the reaction product was galactosamine-1-phosphate. The results indicated that galactosamine phosphorylation is catalyzed by the same enzyme which acts on galactose.

Several compounds which may be related to hexosamine metabolism have been isolated. Three papers by Park (71) reported studies on the compounds which accumulate in *Staphylococcus aureus* by treatment with penicillin. Three substances could be separated and the three contained uridine-5′-pyrophosphate. The rest of the molecule of compound I appeared to be an acetyl amino uronic acid: it gave a positive reaction with the Morgan & Elson reagent and was retained by an anion exchange resin. Compound II contained, in addition, one alanine residue, and compound III, a

peptide containing lysine, glutamic acid and three alanine residues. A related compound has been found in baker's yeast (72). Its structure was found to be identical to that of uridine diphosphate glucose, but with acetylglucosamine instead of glucose.

Careful studies on the Morgan & Elson procedure for the estimation of hexosamines (73) and on the interference produced by sugars and amino acids (74) have been carried out.

Glucuronic acid.—Four papers dealt with the biosynthesis of glucuronic acid studied with C^{14}. Packham & Butler (75) using labelled lactate, pyruvate, and glucose, found that they were incorporated to an equal extent into the glucuronide. Glycerol 1-C^{14} was administered to rats by Doerschuk (76). From the distribution of radioactivity in the bornylglucuronide it was concluded that the biosynthesis occurred by condensation of C_3 intermediates. With 1-C^{14} glucose Eisenberg & Gurin (77) obtained a glucuronide labelled predominantly in the 1-C of the glucuronic acid moiety and concluded that glucose or an equivalent 6 carbon unit is the immediate precursor of glucuronic acid in the rabbit. Essentially the same results with glucose were obtained by Bidder (78). However, when lactate 3-C^{14} was used, practically all the radioactivity appeared in the 6-C of the glucuronide. If the reaction were lactate→glucose→glucuronide, the latter would be expected to be symmetrically labelled. One possibility mentioned by Bidder is that a triose from glucose would give the 1, 2, and 3 carbon atoms of glucuronic acid, and a triose from lactate would give rise to the 4, 5 and 6 carbon atoms.

The fate of uniformly labelled glucuronic acid in normal and in borneal fed guinea pigs was studied by Douglas & King (79). About 50 per cent was excreted unchanged in the urine, about 30 per cent was oxidized to CO_2, and only about 10 per cent was excreted as bornyl glucuronide. It remained undecided whether glucuronide formation was direct or occurred by cleavage of the 6 carbon chain. Ethanol has been found to be partially excreted as ethyl-glucuronide in rabbits (80), and similar results were obtained with methanol.

Miscellanea.—Hastings *et al.* (81) determined the optimum concentration of K^+, Mg^{++} and Ca^{++} cations necessary for glycogen synthesis from glucose and pyruvate in rat liver slices. Brown *et al.* (82) studied the changes of glucose utilization by isolated diaphragm due to the conditions under which the tissue is held prior to incubation.

The phosphorylation of hexoses, pentoses, and glycerol with high energy phosphate compounds and catalyzed by acid phosphatase was studied by Green & Meyerhof (83). The highest rate of transphosphorylation was obtained with acetyl phosphate as donor, and the lowest with phosphopyruvate. Phenylphosphate and phosphocreatine gave intermediate rates. It was concluded that there is no relation between rate of transphosphorylation and energy content of the phosphate donor.

The action of β-hydroxybutyric acid on glucose metabolism in insulin treated eviscerated rabbits was studied by Drury & Wick (84) using glucose-

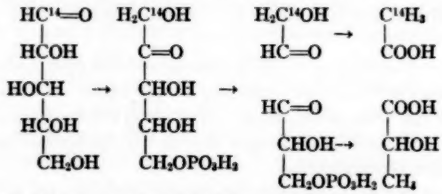
C^{14}. The rate of glucose disappearance was not greatly affected but glucose oxidation, as measured by the formation of $C^{14}O_2$, was markedly reduced. It was concluded that although glucose oxidation in extrahepatic tissues is increased by insulin, another important factor is the presence of other oxidizable substances such as β-hydroxybutyrate, which can enter in competition with glucose. Acetoacetate has been found to inhibit glucose utilization in blood (85).

Meyerhof & Kaplan (86) investigated the mechanism of the cyanide inhibition of yeast fermentation. Being a carbonyl reagent, cyanide can fix glyceraldehyde phosphate and acetaldehyde as cyanhydrins. Cyanide combines with DPN,[2] and it leads to the transformation of pyruvate into its dimer: parapyruvate, which is not decomposed by carboxylase. Aldolase, which has been reported to be a metallo-enzyme, was not inhibited even at 0.1 M concentration, and the most sensitive enzyme was found to be alcohol dehydrogenase. This enzyme does not contain any metal, and therefore it was supposed that cyanide might inhibit it by combining with some carbonyl group.

PENTOSES AND TRIOSES

Pentoses.—An enzyme, xylokinase, which phosphorylates D-xylose with ATP[2] as phosphate donor, was extracted from *Pseudomonas hydrophila* by Hochster & Watson (87). Purified preparations were inactive towards glucose, L-xylose, and other pentoses. As judged by the rate of acid hydrolysis, the reaction product appeared to be D-xylose-5-phosphate.

Further studies on pentose fermentation have strengthened the belief that it takes place by cleavage between carbon atoms 2 and 3. Guest & Lampen (88) used 1-C^{14} xylose in their experiments with *Lactobacillus pentosus.* They observed that the methyl group of the acetic acid formed contained the C^{14}, and postulated a 2-ketopentose as intermediate, as follows:



D-Xylose D-Ribulose-5-phosphate

I

Some evidence was obtained indicating that the initial step is the formation of xylose phosphate and that the compound undergoing cleavage has the ribose configuration.

Xylose fermentation by *E. coli* gives rise to more than one mole of lactic acid per mole of pentose. The process occurs with CO_2 fixation. Nutting &

Carson (89) carried out some tracer studies and found that the carboxyl of lactate may be formed from CO_2 or formate and that the carbon of added C_2 compounds (acetic acid or ethanol) was recovered in the 2 and 3 carbon atoms of lactate. Therefore, this fermentation probably involves a C_2-C_3 split after which the C_3 portion gives lactate and the C_2 portion fixes CO_2 or formate, yielding also lactate.

A C_3-C_2 cleavage of pentose-5-phosphate has been observed with isolated enzyme systems obtained from yeast and animal tissues. An isomerization of ribose-5-phosphate gives rise to ribulose-5-phosphate and the latter then splits to triose-phosphate plus $C_2H_4O_2$ (90, 91, 92). The fate of the unknown product $C_2H_4O_2$ depends on the type of enzymes present in the extract. Thus, Horecker & Smyrniotis (91) added aldolase and obtained sedoheptulose phosphate.

Sedoheptulose was identified by treatment with phosphatase and transformation into sedoheptulosan tetrabenzoate. The triose phosphate was identical with dihydroxyacetone phosphate, as judged by chromatography, but the yield was about half that required for the following reaction:

$$\text{2 Ribose-5-phosphate} \rightarrow \text{2 ribulose-5-phosphate} \rightarrow \text{sedoheptulose phosphate} + \text{triose phosphate} \qquad \mathbf{2.}$$

Evidence was presented indicating that $C_2H_4O_2$ condensed to a tetrose (D-erythrose) which with aldolase and dihydroxyacetone phosphate would give sedoheptulose phosphate.

De La Haba & Racker (92) were able to separate some of the enzymes necessary for the formation of hexose phosphate from ribose phosphate: (*a*) pentose phosphate isomerase; (*b*) the enzyme producing the cleavage of ribulose phosphate to triose phosphate and $C_2H_4O_2$; and (*c*) the enzyme required for the formation of hexose phosphate. The product $C_2H_4O_2$ seems to be different from glycolaldehyde since reactions for this substance are negative (90, 92). Work along a similar line was carried out by Glock (93), who found that about 75 per cent of the ribose phosphate was recovered as hexose monophosphate. This fact and the absence of hexosediphosphate in the extracts exclude hexose monophosphate formation by simple condensation of triose phosphate. Glycolaldehyde was excluded as intermediate.

Sedoheptulose phosphate and ribulose phosphate have been isolated from photosynthetic organisms. Benson *et al.* (94) suggest that these substances are not involved in hexose synthesis, but they may serve as sources of the C_2 units required as CO_2 acceptors. The micro methods of identification used in this work are of interest.

A study of ribose synthesis was carried out by Löw (95). Doubly labelled acetate ($C^{13}H_3 \cdot C^{14}OOH$) was injected to rats and its incorporation in the ribose of polynucleotides was measured. The methyl carbon was found to be incorporated in larger amounts than the carboxyl carbon, and it was concluded that acetate is not a direct precursor of ribose.

Desoxyribose.—The mechanism of synthesis and breakdown of des-

oxyribose phosphate has become clear with Racker's paper (96). An enzyme, desoxyribose phosphate aldolase (DR aldolase), found in microorganisms and animal tissues, catalyses the following reversible reaction:

$$\text{Glyceraldehyde-3-phosphate} + \text{acetaldehyde} \rightleftarrows \text{desoxyribose-5-phosphate} \qquad 3.$$

DR aldolase was partially purified and found to be different from aldolase and from the enzyme catalyzing the breakdown of ribose-5-phosphate.

Phosphoribomutase.—Phosphoglucomutase can act not only on glucose and mannose phosphates, but also on ribose phosphates. Klenow & Larsen (97) found that it catalyzes the reaction ribose-1-phosphate→ribose-5-phosphate, and that glucose-1,6-diphosphate accelerates the reaction. They presented evidence for the occurrence of a transfer, as follows:

$$\begin{aligned}\text{Ribose-1-phosphate} + \text{glucose-1,6-diphosphate} &\rightarrow \text{ribose-1,5-diphosphate}\\ &+ \text{glucose-6-phosphate} \qquad 4.\end{aligned}$$

They did not mention whether the previously described phosphoribomutase (98), which they found to be destroyed by surface denaturation, is different from phosphoglucomutase.

Thiomethylpentose.—Smith & Schlenk (99) devised a method for the estimation of adenine-thiomethylpentose and found that considerable accumulation of this substance takes place when yeast is grown in the presence of methionine. On removal of the methionine the adenine-thiomethylpentose content decreases progressively. Other sulphur compounds could not replace methionine.

Triosekinase.—Lindberg (100) found that glyceraldehyde, dihydroxyacetone and glyceric acid were phosphorylated by kidney homogenates and ATP.[2]

Glyceraldehyde phosphate dehydrogenase.—The mechanism of action of glyceraldehyde phosphate dehydrogenase has been studied by several workers. The theory put forward by Racker & Krimsky (101) starts with the assumption that DPN[2] is combined with an —SH group of the enzyme. The first step would be a cleavage of the bond between DPN and the sulphur of the enzyme by the aldehyde group of the substrate (aldehydolysis). The hydrogen would be transferred to DPN and the acyl group to the sulphur. Thus reduced DPN and a thiol ester would be formed. This ester might then be decomposed by phosphorolysis to form acyl phosphate or by arsenolysis to form a very labile compound which would decompose immediately.

Several types of evidence are consistent with a mechanism of this type. Glutathione was found to be present in the crystalline enzyme and the absorption spectrum of DPN was found to change on combination with the enzyme but not after the —SH groups had been destroyed. Several other facts were presented by Racker & Krimsky (101) in favour of their theory, and Holzer & Holzer (101a), mainly on the basis of experiments with iodoacetate, also reached the conclusion that —SH groups play an important rôle. Moreover, Harting & Velick (102) found that the crystalline enzyme catalyzed the following reactions: (*a*) the exchange of inorganic phosphate

with that of acetyl phosphate; (*b*) the arsenolysis of acetyl phosphate; (*c*) acetyl exchange between acetyl CoA[2] and acetyl phosphate; and (*d*) the acetylation of glutathione. Further evidence that triose phosphate dehydrogenase catalyzes the oxidation of acetaldehyde has also been presented (103).

Evidence against the previous theory which assumed the formation of an arseno-glyceraldehyde phosphate compound has also been obtained by Bucher & Garbade (104) in a careful kinetic study of the action of arsenate on triose phosphate dehydrogenase.

Metabolism of glycerol.—Doerschuk (105) administered 1-C^{14} glycerol to rats and studied the appearance of radioactivity in liver glycogen and in the carcass glycerides. The radioactivity of the glucose was found mainly in positions 1, 3, 4, and 6, and it was concluded that the results were consistent with an initial oxidation and phosphorylation to triose phosphate followed by the known reactions of glycolysis.

Diphosphoglycerate mutase.—Rapoport & Luebering (106) continued their work on the formation of 2,3-diphosphoglyceric acid. A partially purified enzyme from red blood cells was found to catalyze the transformation of 1,3 to 2,3-diphosphoglycerate. Since 3-phosphoglycerate acts as activator, the reaction was formulated as an intermolecular transfer similar to that of other mutases:

$$\text{1,3-diphosphoglycerate} + \text{3-phosphoglycerate} \rightarrow \text{3-phosphoglycerate} + \text{2,3-phosphoglycerate} \qquad 5.$$

Metabolism Beyond the Triose Stage

Tricarboxylic acid cycle.—Enzyme systems catalyzing the reactions of the tricarboxylic cycle (cyclophorase) have been prepared from brain (107), rabbit heart muscle (108), and mammary gland (109). Many organisms have been studied in order to find out whether the tricarboxylic cycle is the main pathway of oxidation. In *Azotobacter* acetate was shown to be incorporated rapidly into citrate, α-ketoglutarate, and succinate (110). Experiments with *Brucella abortus* also indicated the presence of a tricarboxylic acid cycle (111).

Krebs *et al.* (112) have reached the conclusion that the tricarboxylic cycle is not the main pathway of acetate oxidation in yeast. The cells were submitted to freezing and thawing in order to make the cell walls permeable to di- and tricarboxylic acids. Under these conditions malonate inhibited the oxidation of succinate but not that of acetate, and labeled acetate was not appreciably incorporated into the acids of the cycle. In an interesting discussion, the authors insist on the fact that while the necessary enzymes may be present, this does not prove that the cycle is the main oxidation pathway since such reactions may be primarily concerned with the supply of intermediates for other reactions.

Results with *E. coli* were interpreted as showing that the tricarboxylic cycle is not involved in glucose oxidation (113), and according to King &

Cheldelin (114), neither a 6-C nor a 4-C cycle operates to a significant degree in *Acetobacter suboxydans*.

Pyruvate oxidizing enzymes.—Enzymes from several sources have been used in the study of pyruvate oxidation. Korkes, del Campillo & Ochoa (115) obtained a soluble enzyme from heart muscle which catalyzes a diphosphothiamine-dependent oxidative decarboxylation of pyruvate to acetyl CoA[2] and CO_2 in the presence of DPN and CoA as follows:

$$\text{Pyruvate} + \text{DPN} + \text{CoA} \rightarrow \text{acetyl CoA} + CO_2 + \text{DPNH}_2 \qquad 6.$$

The $DPNH_2$[2] produced was reoxidized by another molecule of pyruvate in the presence of lactic dehydrogenase to yield DPN and lactate. With catalytic amounts of CoA the reaction did not progress unless the acetyl CoA formed was decomposed by another system. For instance, with phosphate and transacetylase to yield acetyl phosphate, or with oxalacetate and condensing enzyme to yield citrate. Decomposition of acetyl CoA may also be brought about by CoA-deacetylase, which was found in pig's heart by Gergely *et al.* (116).

The pyruvate oxidase of *Proteus vulgaris* has been separated into two protein fractions which are required for activity. The oxidation was found to proceed in the absence of CoA, but with additional enzymes and CoA, small amounts of acetyl phosphate, acetyl sulphonamide, or citrate were formed (117). One of the *Proteus* proteins appeared to be a pyruvate dehydrogenase requiring diphosphothiamine, and the other an oxidizing system containing cytochrome. No evidence for the intervention of pyruvate oxidation factor was obtained (118).

The complexity of the pyruvate oxidation system is well exemplified in the study of Dolin & Gunsalus (119). Enzymes from *Streptococcus faecalis* were found to catalyze the dismutation of two molecules of α-ketobutyrate to the corresponding acyl phosphate, α-hydroxy acid, and CO_2. With pyruvic acid as substrate there was also formation of acetoin. The system was fractionated into several components: lactic dehydrogenase, phosphotransacetylase, and two other fractions of unknown function. In addition to these four protein fractions, the system required CoA, diphosphothiamine, DPN,[2] phosphate and "pyruvic oxidation factor."

A pyruvate dehydrogenase system obtained from *Clostridium saccharobutycum* was found to require CoA and inorganic phosphate. Acetoacetate was one of the reaction products (120).

From pigeon breast muscle, Jagannathan, Schweet *et al.* (121) obtained a homogeneous preparation of a molecular weight about 4 million, which in the presence of diphosphothiamine oxidizes pyruvate to acetate and CO_2. As oxidant, ferricyanide, oxygen, dyes, or DPN could be used. Acetoin was also formed and supposed to arise from an intermediate produced by the decarboxylation of pyruvate.

Carboxylase.—Singer & Pensky (122) obtained a highly purified preparation of carboxylase from wheat germ. Diphosphothiamin and Mg^{++}, Co^{++},

or Zn^{++} are required for maximal activity. Pyruvate and α-ketobutyrate are decarboxylated at the same rate, and α-ketoglutarate ten times more slowly.

Stoppani & Actis (123) found that yeast carboxylase is inhibited by trivalent organic arsenicals such as methylarsenoxide or mepharsen. Thiol compounds prevented or reversed the effect. The presence of pyruvate greatly decreased the inhibition but diphosphothiamin did not. The enzyme was assumed to contain sluggish reacting thiol groups which would be involved in the formation of the enzyme-substrate complex.

Formation of acetoin.—More information on the various mechanisms of acetoin formation has been obtained. A bacterial system (*Aerobacter aerogenes*) was resolved by Juni (124) into two components, each of which catalyzed one of the reactions:

$$\text{Pyruvate} \rightarrow \alpha\text{-acetolactate} + CO_2 \qquad 7.$$

$$\alpha\text{-Acetolactate} \rightarrow \text{acetoin} + CO_2 \qquad 8.$$

Reaction 7 was stimulated by diphosphothiamine. Besides this system Happold & Spencer (125) obtained evidence for the existence of another system which would form acetoin from acetate with diacetyl as intermediate.

A double mechanism has been postulated by Milhaud *et al.* (126) for the acetoin formation by an extract of *Bacillus subtilis*. With this extract acetoin is formed from pyruvate or acetolactate, but the rate is different according to the age of the original cultures. Manganese ions inhibit when pyruvate is the substrate in contrast with what has been found for the *Aerobacter* and *Streptococcus* enzymes.

Juni (127) also studied the yeast and mammalian systems in which α-acetolactate is not an intermediate. Both systems can form acetoin from pyruvate and acetaldehyde, but they are not identical since only pig's heart can use acetaldehyde alone. The carbonyl carbon of acetoin was found to arise from the carbonyl of pyruvate.

In a study with a highly purified carboxylase from wheat germ, Singer & Pensky (128) found that the relation between the rate of decarboxylation of pyruvate and the rate of acetoin formation remained constant after different purification procedures and also after partially inactivating the enzyme in various manners. They, therefore, concluded that the same enzyme catalyzes acetoin formation and pyruvate decarboxylation. With this enzyme acetoin is formed from pyruvate and acetaldehyde and also from acetaldehyde alone, but at a slower rate. An interesting point is that the reaction product was a mixture of 72 per cent of (+) acetoin and 28 per cent of (−) acetoin. The reaction could not be reversed.

α-Ketoglutarate oxidation.—Sanadi *et al.* (129) obtained from pig's heart an α-ketoglutarate oxidase of molecular weight of about 2 million which was homogeneous as judged by electrophoresis and ultracentrifugation.

DPN[3] and CoA were considered as prosthetic groups of this enzyme which catalyzes the following reaction:

$$\alpha\text{-Ketoglutarate} + \text{DPN} + \text{CoA} \rightarrow \text{succinyl CoA} + CO_2 + \text{DPNH}_2 \qquad 9.$$

The reaction proceeds with catalytic amounts of CoA if coupled with succinyl-CoA deacylase:

$$\text{Succinyl CoA} + H_2O \rightarrow \text{succinate} + \text{CoA} \qquad 10.$$

This enzyme was prepared from pig's heart and highly purified by Gergely *et al.* (116).

The influence of ions on α-ketoglutarate oxidation by mitochondria was investigated by Hartman & Kalnitsky (130). The addition of Mg^{++} or Cu^{++} after Mn^{++} had no influence on the oxidation, but if added before there was a marked inhibition.

The rôle of CoA[2].—This point was expertly reviewed by Ochoa & Stern in last year's *Annual Review of Biochemistry*. An important development has been the elucidation of the mechanism by which acetyl CoA is synthesized from free acetate and CoA as follows:

$$\text{ATP} + \text{CoA} \rightleftharpoons \text{AMP} + \text{CoA pyrophosphate} \qquad 11.$$

$$\text{CoA pyrophosphate} + \text{acetate} \rightleftharpoons \text{acetyl CoA} + \text{pyrophosphate} \qquad 12.$$

Lipmann *et al.* (131) measured the over-all reaction 11 plus 12 by using hydroxylamine as acetyl acceptor from acetyl CoA. With crude enzymes containing pyrophosphatase, phosphate appeared as inorganic, but when a purified yeast enzyme was used pyrophosphate accumulated.

The reversion of reaction 12 was detected by estimating the disappearance of acetyl CoA which only occurred when inorganic pyrophosphate was added, but not with phosphate or arsenate. The equilibrium of reaction 12 was found to be displaced to the right. However, starting with CoA, acetyl phosphate, and phosphotransacetylase as acetyl CoA feeders, plus inorganic pyrophosphate and AMP[2], considerable amounts of ATP were formed by reversal of reaction 11 plus 12. Some properties of CoA pyrophosphate were described: it is stable to acid at room temperature and may be heated at neutral reaction with only slight decomposition.

Evidence confirming Lynen's work, which showed that the acetyl group combines with the —SH group of CoA, was presented by Stern, Ochoa & Lynen (132). The synthesis of citrate from acetyl CoA and oxalacetate in the presence of crystalline condensing enzyme was found to be accompanied by the appearance of stoichiometric amounts of —SH groups. From the equilibrium values of the reaction at different pH values when coupled with the malic enzyme, it was deduced that the free energy of the bond in acetyl CoA is of the order of 10,000 cal. per mole.

Besides the reactions in which CoA[2] acts as an acetylating agent, that is transferring an CH_3CO-group, it acts by transferring a —CH_2COOH group to oxalacetate to yield citrate. Weinhouse (133) has assumed that acetyl CoA may lose a proton as follows:

$$CH_3{-}\overset{\overset{\displaystyle O}{\|}}{C}{-}S{-}R \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} CH_2{=}\overset{\overset{\displaystyle O^-}{|}}{C}{-}SR \rightleftharpoons \bar{C}H_2{-}\overset{\overset{\displaystyle O}{\|}}{C}{-}SR, \qquad 13.$$

giving two resonating forms of the ester anion, and that these two structures might account for the experimental observations indicating the existence of two forms of active acetyl. He further developed the thesis to explain β-oxidation of fatty acids.

The purification of the enzyme from pigeon's liver, which catalyzes the transfer of acetyl from acetyl CoA to arylamines, was described by Chou & Lipmann (134).

Stadtman (135) detected the following enzymes in extracts of *Clostridium kluyverii:*

$$\text{Acetyl phosphate} + \text{CoA} \rightleftharpoons \text{acetyl CoA} + \text{inorganic phosphate} \quad \text{(phosphotransacetylase)} \qquad 14.$$

$$\text{Acetyl CoA} + \text{propionate} \rightleftharpoons \text{propionyl CoA} + \text{acetate} \quad \text{(CoA transpherase)} \qquad 15.$$

$$\text{Propionyl CoA} + \text{inorganic phosphate} \rightleftharpoons \text{propionyl phosphate} + \text{CoA} \qquad 16.$$

The sum of these equations results in the transfer of phosphate from acetylphosphate to propionate.

Phosphotransacetylase (reaction 14) was purified and found to require potassium or ammonium ions (136). Activity measurements were carried out by estimating the disappearance of acetyl phosphate in the presence of arsenate and catalytic amounts of CoA.[2] That is, reaction 14 plus 17.

$$\text{Acetyl CoA} + \text{arsenate} \rightleftharpoons \text{acetate} + \text{CoA} + \text{arsenate} \qquad 17.$$

The ΔF for reaction 14 was found to be -3000 calories, and some properties of acetyl CoA formed by transacetylase were studied (136). A nonenzymatic transfer of acetyl from acetyl CoA to other mercaptans was detected.

Extracts of *Clostridium kluyverii* catalyze the oxidation of acetaldehyde with DPN[2] or TPN and CoA to acetyl phosphate. Burton (137) could separate two enzymes. One was phosphotransacetylase and the other catalyzed the reaction:

$$\text{Acetaldehyde} + \text{CoA} + \text{DPN} \rightarrow \text{acetyl CoA} + \text{DPNH}_2 \qquad 18.$$

Nachmansohn *et al.* (138) prepared the enzyme from pigeon's liver which in the presence of ATP[2] and acetate catalyzes the formation of acetyl CoA, that is, the over-all reaction 11 plus 12. They reported the finding that ATP+ acetate could be replaced by thiolacetate, so that thiolacetate with catalytic amounts of CoA could acetylate choline (reactions 19 plus 20) or sulfanilamide.

$$\text{Thiolacetate} + \text{CoA} \rightleftharpoons \text{acetyl CoA} + H_2S \qquad 19.$$

$$\text{Acetyl CoA} + \text{choline} \rightleftharpoons \text{acetyl choline} + \text{CoA} \qquad 20.$$

Some enzyme preparations catalyzed both reactions 19 and 20, while one obtained from squid ganglion only catalyzed reaction 20.

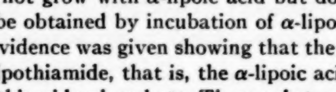
The squid ganglion enzyme was further tested with catalytic amounts of CoA and acetyl feeding reactions such as the phosphotransacetylase and

pyruvate oxidation systems and also with citrate plus condensing enzyme. In each case acetyl choline was formed (139). With choline and acetyl CoA as substrates equal amounts of acetyl choline and of —SH groups were found to be formed.

Coenzyme A.—Two methods of preparation have been published. One starts with the fermentation products of *Streptomyces fradiae* (140) and the other with dried brewer's yeast (141). In both products the analytical data agreed with that of a substance formed by combination of adenosine, three phosphate groups, pantothenic acid, and thioethanolamine. A method for the acetylation of CoA[2] with thiol acetate was described by Wilson (142). Gregory & Lipmann (143) have shown that CoA can combine with other —SH containing substances, thus making purification difficult. They therefore propose a method in which reducing agents are used.

The position of the phosphate which esterifies the adenylic acid moiety but does not form part of the pyrophosphate linkage was studied by Wang, Shuster & Kaplan (144) using an enzyme extracted from barley. This enzyme (*b*-nucleotidase) splits "*b*-nucleotides" and inactivates CoA, but does not act on TPN.[2] Moreover, treatment of TPN and CoA with pyrophosphatase leads to the formation of two different adenosinediphosphates. Treatment of the adenosinediphosphate from CoA with "*b*-nucleotidase" gave adenosine-5′-phosphate, whilst treatment of the corresponding TPN degradation product did not. From this and other evidence, it was concluded that the monoester phosphate in CoA is in the *b* form, as contrasted to TPN which is a derivative of adenylic acid *a*. Studies by periodate oxidation have confirmed the fact that the nonpyrophosphate phosphate group occupies position 2′ or 3′ of the adenosine residue (145).

Pyruvate oxidation factor.—Studies on the structure of α-lipoic, thioctic acid, or protogen A, the catalytic agent required for oxidative decarboxylation of pyruvic acid, have shown that it is an intramolecular disulfide of dimercapto-n-octanoic acid (146, 147, 148). The suggested structure is as follows:



II

Several other compounds with similar catalytic properties have been found in natural products, for instance, Gunsalus *et al.* (149) reported at least four different compounds. Reed & De Busk (150) obtained an *E. coli* mutant which does not grow with α-lipoic acid but does so with combined forms which could be obtained by incubation of α-lipoic acid and thiamine with *E. coli*. Good evidence was given showing that the combined forms corresponded: one to lipothiamide, that is, the α-lipoic acid amide of thiamine and another to lipothiamide phosphate. These substances were required for the oxidation of pyruvate or α-ketoglutarate by the *E. coli* mutants. Another interesting point is that the mutants could grow without lipothiamide when

acetate, citrate, and succinate were present. A method for the estimation of the pyruvate oxidation factor using *Streptococcus faecalis* was published by Gunsalus *et al.* (151).

Diphosphothiamine.—An enzyme from rat liver which phosphorylates thiamine was purified by Leuthardt & Nielsen (152). Besides Mg^{++}, a great excess of ATP[2] was required for maximal velocity. AMP and ADP acted as inhibitors, and since thiamine monophosphate was not phosphorylated, it was concluded that the reaction consists in a transfer of a pyrophosphate group from ATP to thiamine. A protein fraction obtained from yeast by Steyn-Parvé (153) was found to catalyze the phosphorylation of thiamine and also of the monophosphate, although at a slower rate.

Utilization of pyruvate and acetate by yeast.—Wang & co-workers (154) have studied the utilization of carboxyl-labelled acetate and carbonyl-labelled pyruvate by yeast. Pyruvate could not be metabolized in the presence of glucose, though it was readily assimilated when the yeast was first grown on glucose and then transferred to a pyruvate containing medium. Acetate did not sustain the growth of yeast, but about one-tenth of the total radioactivity was incorporated into the yeast, thus indicating a considerable exchange with intermediary metabolites. About one-third of the pyruvate was assimilated by the yeast cells. The difference in the pattern of utilization of both compounds became apparent when samples of the yeast were fractionated and the labelling of different compounds was examined (155). The isotopic labelling from pyruvate was relatively uniform in the fat and protein fraction and less in steroids and humin. Labelling from acetate was about fourfold greater in the fats than in proteins, and the activity of fats from acetate was about twice as high as in those derived from pyruvate. The labelling of the aspartate from acetate (156) suggested a C_2-C_2 condensation and with pyruvate as substrate a C_3-C_1 condensation according to a Wood & Werkman or "malic" fixation scheme.

Fumaric acid.—Massey (157) reported the crystallization of fumarase. The enzyme was about ten times more active than the previously described crystalline fumarase, and appeared homogeneous on electrophoresis and sedimentation.

Oxalacetic acid.—Byerrum & Rothschild (158) studied the influence of pH and ionic strength on the decarboxylation of oxalacetate by an enzyme from *Cucurbita pepo.* The decrease in activity between pH 4.2 and 5.0 was compared with the solubility of the protein in the same pH range. The two were found to parallel one another closely. The increase in activity between pH 3.5 and 4.2 was postulated to be the result of the increasing concentration of the oxalacetate bivalent anion and the decreasing concentration of univalent anion. The latter would exert an inhibiting action.

Citric acid.—Bornstein & Johnson (159) investigated the formation of citrate by *Aspergillus niger.* It was concluded that it is probably formed by condensation of "active acetate" and a C_4 dicarboxylic acid arising mainly by condensation of carbon dioxide with a 3-carbon compound. This CO_2

fixation was not belived to be a simple reversal of oxaloacetate decarboxylation. As to the origin of the oxalate formed in the fermentation, it was considered to be formed by splitting of oxalacetate or oxalosuccinate, but not by direct oxidation of acetate.

The decomposition of citrate which occurs on anaerobic incubation with liver homogenates was studied by Martius & Nitz-Litzow (160) and found to consist in an oxidation to α-ketoglutaric acid coupled with a reduction of the latter to α-hydroxyglutaric acid.

The disturbance of citric metabolism produced by fluoroacetate was discussed in an interesting lecture by Peters (161). Fluoroacetate, which is not toxic, as such has been found to be transformed enzymically into a substance believed to be fluorocitrate (162, 163, 164). The fluorotricarboxylic acid formed is a potent inhibitor of aconitase, however, the concentration needed for inhibition of the soluble enzyme is much higher than for homogenates. According to Peters, this discrepancy may be due to the fact that in homogenates the enzymes which catalyze the reactions of the tricarboxylic acid cycle are contained in the mitochondria, which behave as slightly permeable boxes. Fluoroacetate may enter the box, but the fluorocitrate formed will not leave it so readily. Since it is not oxidized, it will accumulate and produce a greater effect than on soluble enzyme systems. Kandel & Chenoweth (165) also discussed the mechanism of fluoroacetate poisoning and reported experiments showing that the toxic symptoms are not due to the accumulation of citrate.

Lactate oxidation.—Two lactic oxidases were obtained from *Mycobacterium tuberculosis aviarum* by Yamamura *et al.* (166). One oxidizes lactate to acetate directly with oxygen and was supposed to contain flavin adenine dinucleotide. It does not act on pyruvate. The other oxidase gives pyruvate as oxidation product, requires DPN,[2] and was found to be similar to mammalian lactic dehydrogenase.

An electrophoretic investigation of Straub's crystalline dehydrogenase was carried out by Neilands (167), who observed two components, both of which were active. Schweit & Hakala (168) have carried out a kinetic study.

Propionate metabolism.—It has been assumed that propionate arises from glucose by cleavage to two C_3 fragments. These may be reduced to propionate or transformed by CO_2 fixation to succinate, which would yield propionate by decarboxylation. Wood & Leaver (169) have carried out experiments on *Propionibacterium arabinosum* with two types of radioactive glucose. The results excluded succinate as the sole precursor of propionate.

The conversion of lactate to propionate appears to be different in *Clostridium propionicum* from that in other propionic acid bacteria. Johns (170) has presented evidence indicating that acrylate is an intermediate.

Investigations with labelled propionate in rats have shown that propionate is as effective a precursor of acetyl groups as pyruvate (171). However, propionate seemed to traverse a route involving a symmetrical dicarboxylic acid while pyruvate was so routed only in a small proportion.

The formation of a symmetrical intermediate from propionate was also indicated by the experiments of Daus *et al.* (172). Besides being incorporated into glucose, lactate, alanine, etc., the carbon atoms of propionate were found in β-hydroxyvaleric acid. From the distribution of the labels it was deduced that β-hydroxyvaleric acid is formed by direct propionlylation of a 2-carbon compound in a manner similar to the acetylation which leads to acetoacetate.

Glyoxal.—Kun (173) found glyoxal to be a strong inhibitor of hexokinase and triose phosphate dehydrogenase. It also inhibited the respiration of brain, kidney and heart slices, but not that of liver slices. Tissue extracts were found to convert glyoxal anaerobically to glycollic acid when reduced glutathione was added. The diglutathione complex of glyoxal was readily converted to glycollic acid while the methyl glyoxal complex was not. Glycollic acid was oxidized to glyoxyllic by a soluble liver enzyme reacting directly with molecular oxygen.

Formaldehyde and formic acid.—The precursors of formate in rats have been investigated by Weinhouse & Friedmann (174) by administration of labelled compounds together with formate. The labelling of the formate excreted in urine was subsequently measured. In this manner, the following groups were found to be transformed into formate: the α-carbon of glycine, the α-carbon of glycollate and glyoxalate, the β-carbon of serine, the methyl groups of acetone, sarcosine, and methionine. Results were negative with the carboxyl of glycine and with glucose.

In *Rhizopus* formate was found to act as precursor of the methyl group of lactic acid and ethanol and of the methylene groups of fumarate (175). In *Aspergillus niger*, which is believed to form citrate by the known reactions of the tricarboxylic cycle, labelled formate was incorporated into the citrate and the distribution of the label indicates that formate is first oxidized to CO_2 (176).

An enzyme from mammalian liver: formaldehyde-pyruvic acid carboligase, has been found to condense pyruvate and formaldehyde to an unidentified product (177, 178). Formaldehyde could not be replaced by other aldehydes and phenylpyruvate could be used instead of pyruvate. A related process was found to occur upon incubation of a liver enzyme, radioactive formaldehyde and dihydroxyacetone or fructose diphosphate (179). The radioactivity was recovered in pentoses and hexoses, and mostly in an unknown compound believed to be an aldotetrose. The enzyme was stated to be different from aldolase.

CO_2 fixation mechanisms.—Horecker & Smyrniotis (180) have established the reversibility of the reaction catalyzed by 6-phosphogluconic dehydrogenase:

$$\text{6-Phosphogluconate} + \text{TPN} \rightleftharpoons \text{ribulose-5-phosphate} + CO_2 + \text{TPNH}_2 \qquad 21.$$

and have thus discovered a case of C_1 plus C_5 addition. They consider that the reaction is similar to the oxidative decarboxylation of isocitrate to α-ketoglutarate and of malate to pyruvate. All three reactions can be

regarded as oxidative decarboxylations of β-hydroxy acids. In connection with this condensation reaction it is interesting to mention that Benson *et al.* (94) found ribulose-5-phosphate and the corresponding diphosphate among the products of photosynthesis in green plants.

A C_2 plus C_1 condensation occurs during the fermentation of xylose by *E. coli*. It has been found (89) that 1.3 to 1.4 moles of lactate are formed per mole of xylose. An explanation accounting for the excess of lactate would be a C_2, C_3 split followed by CO_2 fixation on C_2.

The metabolic pathway of CO_2 in *Clostridium thermoaceticum* was investigated by Wood (181). This organism ferments glucose to acetate, and in the presence of labelled CO_2 approximately equal amounts of methyl and carboxyl labelled acetate are formed. The problem of differentiating a mixture of doubly labelled and normal acetate from one of methyl and carboxyl singly labelled acetate was solved by converting the C^{13}-acetate to ethylene and analyzing it with a mass spectrometer. It was established that a substantial amount of the acetate of the fermentation is doubly labelled and that approximately equal amounts are carboxyl-labelled. It was concluded that the acetate is formed by two processes: (*a*) from glucose, probably without CO_2 fixation, and (*b*) by a synthesis in which the two carbons are derived from CO_2 with little or no dilution, and which does not necessarily involve direct combination of CO_2. The major part of the singly labelled acid was believed to be formed by an exchange reaction of CO_2 with the carboxyl of acetate.

CO_2 fixation in photosynthesis.—Cell free spinach leaf macerates, which exhibit an increase in CO_2 fixation upon illumination, were prepared by Fager (182). More than 70 per cent of the increment of fixation due to light was found in phosphoglyceric and pyruvic acids. The increment was small but reproducible. After a one-minute experiment with labelled CO_2, the ratio of specific activities in phosphoglyceric and pyruvic acids was 10 to 1, and after 15 minutes it was 3 to 1. The 10 to 1 relation in short experiments indicates that phosphoglycerate is formed before pyruvate. Moreover, during illumination part of the phosphoglycerate was transformed into sugars. Addition of glyoxal or 2-phosphoglycolaldehyde increased the amount of CO_2 fixed in the light without increasing dark fixation, and it was suggested that a compound closely related to these substances functions as a two-carbon acceptor for CO_2. The leaf macerate was prepared at low temperature and under relatively anaerobic conditions. It behaved differently from preparations used by other workers in that no oxygen was evolved in the light, even in the presence of oxidants.

Fager & Rosenberg (183) have endeavoured to find out whether the rapid photosynthetic fixation of $C^{14}O$ in phosphoglyceric acid can be due to an exchange reaction unrelated to the photosynthetic sequence. This possibility, which had been suggested by Utter & Wood (184) in a recent review, was investigated by kinetic studies in which total CO_2 fixation in short periods of photosynthesis was measured simultaneously with fixation in

phosphoglycerate. It was concluded that labelling of phosphoglycerate is a step in the photosynthetic process and not due to an independent exchange reaction.

The interesting experiments of Ochoa & Vishniac (185, 186) on the photosynthetic reduction of pyridine nucleotides by green grana have been continued. The photosynthetic process could thus be linked to CO_2 fixing enzyme systems as follows:

$$H_2O + TPN\ (\text{or DPN}) \xrightarrow{\text{light}} TPNH_2(\text{or } DPNH_2) + 1/2\ O_2 \qquad 22.$$

$$CO_2 + \text{pyruvate} + TPNH_2 \xrightarrow{Mn^{++}} \text{L-malate} + TPN \qquad 23.$$

$$CO_2 + \alpha\text{-ketoglutarate} + TPNH_2 \xrightarrow{Mn^{++}} \text{D-isocitrate} + TPN \qquad 24.$$

CO_2 could be fixed on pyruvate with the "malic enzyme" (equation 23) or on α-ketoglutarate (equation 24) with isocitric dehydrogenase. With appropriate enzymes other reactions could be coupled with the photochemical reduction of DPN,[2] such as the reduction of pyruvate to lactate, the reductive amination of α-ketoglutarate to glutamate. The formation of hexose diphosphate could be obtained from phosphoglycerate and ATP by reversal of the known glycolytic reactions.

When grana were supplemented with an extract of *E. coli*, fumarate could be reduced to succinate, and if further supplemented with TPN,[2] "malic enzyme," and fumarase, a photochemical formation of succinate from pyruvate and CO_2 could be obtained. The rate of the photochemical reactions was in general low as compared with those of intact plants, a fact which may be due to the incomplete knowledge of the factors involved in hydrogen transfer to the pyridine nucleotides. Ochoa & Vishniac consider that their experiments support the view that CO_2 fixation occurs by analogous mechanisms in all cells, although there exists a diversity of energy sources.

Hormones

Diabetes.—Siliprandi & Siliprandi (187) presented further evidence indicating that in diabetes the phosphorylation of thiamine to the diphosphate is impaired. They studied the liver disphosphothiamine increase in alloxan diabetic rats after thiamine injection. The increase was found to be much lower than in normal rats. Some results in human diabetics were consistent with their theory (188). However, van Marken *et al.* (189) were unable to confirm the results in human diabetics nor were they able to detect an increase in blood pyruvate in diabetic coma. They attribute previous results to interference by acetoacetate.

Martinez (190) observed that many sulphur containing compounds decreased the incidence of pancreatic and especially, alloxan diabetes, and produced an increase in —SH content of tissues. It was confirmed that diabetes can be produced in rabbits with 8-hydroxyquinoline (191). The action is

increased by simultaneous administration of methylene blue and is additive with that of alloxan.

Diet was found to influence the diabetogenic action of alloxan. In rats fed with different fats and oils it was observed that the higher the degree of unsaturation of the fatty acids, the higher was the incidence of diabetes (192, 193).

Houssay (194) and Rodriguez (195) reported that administration of variable amounts (15 to 50 μg. per day) of oestrogens decreased the frequency of diabetes in partially pancreatectomized rats. A hypertrophy and hyperplasia of the Langerhans' islets was concomitantly observed, and the protective action of oestrogens was found to be maintained after administration was discontinued. Alloxan diabetes disappeared after combined treatment with insulin and oestradiol in about 70 per cent of the cases. The effect was not produced by insulin alone, and only partially with oestrogens alone (193).

In the hereditary obesity-diabetes syndrome of mice, Guggenheim & Mayer (196) observed that the main biochemical lesion is a partial blocking of the oxidation of the C_2-units with an increase in lipogenesis.

Insulin.—Chaikoff and co-workers (197) compared lipogenesis from labelled acetate, lactate, or glucose in liver slices of normal or diabetic rats that were fed for four days with glucose or fructose. In glucose fed diabetic rats lipogenesis from acetate and lactate was greatly depressed. In the fructose fed fats lipogenesis was normal. However, fructose feeding did not correct the defective lipogenesis from C^{14}-glucose. This is in contrast to the action of insulin which restores lipogenesis, not only from lactate and acetate, but also from glucose. A high activity of the glycolytic system was assumed to be required for maintenance of lipogenesis in the liver.

A second paper (198) dealt with labelled acetate utilization by liver slices of fasted rats. Addition of insulin alone had no effect on lipogenesis or CO_2 formation from acetate. Glucose alone increased lipogenesis and glucose plus insulin resulted in still higher values. It was concluded that the action of insulin on lipogenesis is probably a secondary effect to its priming of glucose utilization.

Another investigation (199) referred to the conversion of C^{14}-acetate to glucose by liver slices of normal, diabetic, and insulin-treated diabetic rats. In diabetic-insulin treated livers the incorporation of acetate into glucose and the $C^{14}O_2$ recoveries were greatly reduced. The effect of insulin was attributed to a shift of acetate to fatty acid synthesis which would reduce the amount of acetate entering the tricarboxyllic cycle which leads to glucose synthesis and CO_2 formation.

Haugaard & Marsh (200) found that insulin "in vitro" increased the oxygen uptake of retroperitoneal adipose tissue in the presence of glucose, lactate, succinate, pyruvate, or acetate. The respiratory quotient was decreased in the presence of pyruvate, but not with other substrates.

The results of Villee *et al.* (201) on the utilization of radioactive glucose

and pyruvate by rat diaphragm "in vitro" accord with the theory that insulin produces a stimulation of the hexokinase system and that it has an additional effect on one or more reactions involved in the condensation of pyruvate with oxalacetate. This latter action appears to be antagonized by adrenal hormones.

Diaphragms from normal, pancreatectomized, or adrenolectomized rats were used. In every case insulin increased glucose utilization, glycogen synthesis, and pyruvate formation from glucose. Furthermore, insulin normalized pyruvate uptake in diaphragms from diabetic animals, but did not affect that of normal or adrenalectomized animals.

Insulin has been found to increase the passage of glucose from blood to the intraocular fluid (202). It was suggested that an enzyme system present in the blood-aqueous layer determines the mobilization of glucose.

Confirmatory evidence of the antagonism between adrenal cortex and insulin on hexokinase was obtained by Teng *et al.* (203). Studies with isolated rat diaphragm (204) showed that even with very low levels of ATP[2] the rate of glucose uptake was not diminished and could still be increased by insulin or by a higher glucose concentration. Therefore, ATP concentration did not appear to be a limiting factor and the effect of insulin was attributed to a direct action on hexokinase.

The combination of insulin with adipose tissue (200) and also with slices of mammary gland has been studied (205). The complex formed was found to withstand repeated washings. Its action consists in increasing the respiratory quotient, probably by increasing fat synthesis from added carbohydrate or acetate. Lactose synthesis "in vitro" was not affected by insulin.

Pituitary.—The action of the growth hormone on the glucose uptake of the isolated diaphragm of rats was studied by Park *et al.* (206). Crude or purified preparations had a biphasic action. First they produced a lowering of blood sugar concentration and a stimulation of glucose uptake by the diaphragm. In a second phase which occurred two to three hours after the injection, there was an inhibition of the glucose uptake by diaphragm and blood sugar returned to normal values.

It was concluded that the same principle produces the stimulatory and inhibitory effects, and that the stimulatory action is a direct effect of the hormone, but that the inhibitory effect is indirect and depends on the modification "in vivo" of the crystalline protein into a directly inhibitory principle The stimulatory action could be obtained by "in vitro" addition of growth hormone, but the inhibitory action could not be obtained "in vitro" and only became apparent some hours after injection in the whole animal.

Steroids.—The general initial effect of oxygenated steroids is to increase total carbohydrate, especially liver glycogen. Hyperglycemia and glycosuria have been observed and attributed to an enhancement of gluconeogenesis from noncarbohydrate precursors. This point has been investigated by Welt *et al.* (207) using a method which consists in injecting C^{14}-glucose at a constant rate, and then comparing the specific activity of administered and ex-

creted glucose. The glucose production from noncarbohydrate precursors can thus be estimated. Treatment with cortisone resulted in a sevenfold increase in gluconeogenesis with no great change in glucose oxidation. In alloxan diabetic rats the rate of gluconeogenesis was doubled. The results of Cohn *et al.* (208) also showed that the main action of ketosteroids is to increase gluconeogenesis from proteins.

After large doses of corticosterone, cortisone, or pregnenolone, the total carbohydrates and the glycogen of brain were found to be increased (209), and the changes were attributed to an inhibition of oxidation.

Miscellaneous.—A glycogenolytic factor was extracted from hog liver (210). It produces a decrease in liver glycogen in rats with no changes in muscle glycogen. Its action is different and independent from that of adrenalin; it is dializable and is not a protein or a lipid.

Reviews

The following reviews on metabolism were published: Nervous tissue by Coxon (211); Semen by Mann (212); Heart muscle by Green (213); Ethanol by Jacobsen (214); Formate by Wood (215); Cyclophorase by Green (216); Acetyl CoA[2] by Holzer (217); Hyperglycemic factor of pancreas by Pincus (218).

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LIPID METABOLISM[1,2]

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In view of recommendation by the Editors that a critical evaluation should be attempted rather than a complete synopsis of this year's work, only a few aspects of lipid metabolism have been covered, and even here, selection has been necessary. The following subjects were abstracted but later omitted: nutritional value of fat, essential fatty acids, synthesis and metabolism of choline, choline oxidase, lipoproteins in human disease, acetoacetate formation and ketosis, obesity, lipid composition in tissue, cholesterol esterification, analytical procedures, and, to a large extent, also action of hormones and vitamins.

DIGESTION AND ABSORPTION OF LIPIDS

This topic has been given specific attention in Volume 21 of this series [Frazer (1)] and elsewhere [Desnuelle (2); Favarger (3)]. Frazer's views have been presented again (4) and applied to the interpretation of the absorption defect in sprue and other steatorrhoeas (5, 6).

The original observations by Artom & Reale that mono- and diglycerides are formed in both the digestion (7) and the synthesis (8) of fats by pancreatic lipase have been amply confirmed. A preferential splitting of the 1,3 ester linkages with formation of 2-monoglycerides occurs in the pancreatic digestion of triglycerides of higher fatty acids (9), in that of the triglycerides of lower fatty acids 2,3-diglycerides are chiefly formed [Schønheyder & Volqvartz (10)]. After fatty meals, the lipids in the intestinal lumen contained 37 per cent to 50 per cent of monoglycerides [humans: Kuhrt *et al.* (10a)], or 16 per cent of mono- and 36 per cent of diglycerides [rats: Mattson *et al.* (10b)]. However, both *in vitro* and *in vivo*, the relative proportion of the various products formed in the digestion of triglycerides is markedly affected by the presence of Ca^{++} and bile salts [Desnuelle & Constantin (11)]. Experiments with C^{14}-palmitate indicate that, simultaneously with the hydrolysis, a resynthesis of the free fatty acid with the mono- and diglycerides occurs [Borgström (12)].

From the analysis of the chyle of rats receiving triglycerides doubly labeled in the glycerol and fatty acid moieties, Reiser *et al.* (13) calculated that half or more of the fatty acids were absorbed as monoglycerides. A large portion of the labeled glycerol was metabolized, indicating that complete hydrolysis of the triglycerides also took place. Both free fatty acids and

[1] The main period covered by this review is December 1951 to November 1952.

[2] The following abbreviations are used: ADP for adenosine diphosphate; ATP for adenosinetriphosphate; CoA for coenzyme A; DME for dimethylethanolamine; DPN for diphosphopyridine nucleotide.

monoglycerides were utilized in the synthesis of phospholipids [Reiser *et al.* (13)]. The occurrence of a partial synthesis with consequent redistribution of the labeled fatty acids (12) makes these calculations rather uncertain. On the other hand, additional evidence for the possibility of a particulate absorption is supplied by the results obtained after ingestion of mineral oil [Bernhard & Scheitlin (14)], or labeled hydrocarbons [see below: (83)]. Bergström, Borgström & Rottenberg (15) and Borgström (16) fed labeled fatty acids, either dissolved in corn oil or incorporated into corn oil triglycerides by transesterification. Except for a slower absorption of the free fatty acids, no differences were found in the isotope distribution of the lymph fatty acid or in the rate of $C^{14}O_2$ elimination. Stearate was incorporated into the phospholipids of the intestine to a larger extent than palmitate or myristate. Marked differences were found in the absorption of various labeled fatty acids from different sections of the small intestine [Favarger (17)]. At present it appears, therefore, that fats can be absorbed under various forms (free fatty acid, mono-, di-, and triglycerides) and that the relative proportion of each of these forms may vary, depending upon the anatomical location, upon the fatty acid composition, and especially upon a number of intraluminar factors. The importance of bile for fat absorption has been shown again in experiments on dogs with bile fistula [Searle & Annegers (18)]. The action of bile salts (and probably also that of lecithin and choline) does not seem to be due merely to an improved emulsification, since various synthetic emulsifiers were ineffective [Tidwell & Nagler (19); Annegers (20)].

As for the pathways of absorption, simultaneous analyses were carried out on the plasma from the portal and the inferior vena cava of rats receiving either C^{14}-palmitate or C^{14}-decanoate. The results indicated that, unlike the long-chain fatty acid, a large proportion of the C_{10} acid was transported by the portal route [Kiyasu, Bloom & Chaikoff (21)]. Tritium- or C^{14}-cholesterol, fed to rats, was almost quantitatively recovered in the lymph of the thoracic duct where about 50 per cent was present as cholesterol esters [Biggs, Friedman & Byers (22); Chaikoff *et al.* (23)]. Synthesis of these esters occurs possibly in the lymph itself, since esterification of cholesterol in the intestinal mucosa could not be demonstrated. Esters of D-labeled cholesterol were rapidly hydrolyzed. Cholesterol absorption paralleled that of fats [Favarger & Metzger (24)]. However, when bile was excluded from the intestine, small amounts of palmitate were still absorbed, whereas no cholesterol was absorbed [Siperstein, Chaikoff & Reinhardt (25)]. According to Peterson (26) and Peterson, Nichols & Shneour (27), plant sterols, which are not absorbed, interfere with cholesterol absorption.

FATTY ACID METABOLISM[3]

SYNTHESIS

Liver.—As reported previously (29), cell-free preparations of pigeon liver synthesize fatty acids from labeled acetate. The enzyme system can be

[3] Several papers on this topic published between October 1951 and February 1952 were included in a previous review [Bloch (28)] and are not mentioned here.

extracted from the acetone powder of liver mitochondria but requires addition of the original supernatant for activity. Aerobically the process is stimulated by Mg^{++}, DPN,[2] cytochrome-*c*, and very markedly by citrate. Surprisingly enough C^{14}-acetyl CoA seemed to be a less efficient precursor of fatty acids than C^{14}-acetate. Anaerobiosis affected fatty acid synthesis only slightly, but oxidation of octanoate to acetoacetate or CO_2 was practically suppressed [Brady & Gurin (30)].

In liver slices lipogenesis from C^{14}-acetate is strikingly reduced by previous fasting or by a caloric restriction in the diet of the animals. Refeeding after fasting or even a single administration of glucose enhances fatty acid synthesis to a level higher than that found in the tissue of animals on ordinary diets. *In vitro* addition of insulin did not affect fatty acid synthesis or oxidation of acetate to CO_2. Glucose augmented both processes, but with glucose plus insulin lipogenesis was greater than with glucose alone [Lyon, Masri & Chaikoff (31); Masri, Lyon & Chaikoff (32); Medes, Thomas & Weinhouse (33)]. The similarity in the utilization of acetate by fasted and diabetic livers is striking, since in both conditions fatty acid synthesis is depressed, whereas formation of acetoacetate and cholesterol and acetate oxidation are not. The most likely explanation is that lipogenesis depends upon an active glycolysis (31), and it is impaired by a diminished availability of the substrates for glycolysis (fasting) as well as by a defect in the utilization of the available substrates (diabetes). The finding that with the soluble enzyme from pigeon liver synthesis of fatty acid occurs even in anaerobiosis is not incompatible with this interpretation, since glycolysis still proceeds in aerobiosis and may supply the energy required for lipogenesis. Of course, in this process, a continuous source of hydrogen is also required; reduced DPN[2] is suggested tentatively as such a source (30). A decrease in fatty acid synthesis caused by fasting could be demonstrated in slices of tissues other than liver (33) and also in intact rats injected with C^{14}-acetate [Van Bruggen *et al.* (34); Coniglio, Anderson & Robinson (35)].

Mammary glands.—Previous results indicate that the mammary glands of rabbits, rats, and goats synthesize fat very actively from small molecules and are reviewed by Popják (36). The cow is about as efficient as the goat in using acetate-C for fat formation [Kleiber *et al.* (37)]. Suggestive evidence that this process might be visualized as a reversal of the beta-oxidation is offered by the isotope distribution in the short-chain fatty acids which were isolated from the milk of goats which had received acetate-1-C^{14}. In this study a method of stepwise degradation of fatty acids was used by which two carbons are removed from the carboxyl end of the chain [Hunter & Popják (38)]. As expected, only the odd numbered carbons in the fatty acids were radioactive. The relations between the specific activities of these carbons were as follows: in butyrate, $C_1 = C_3$, in caproate, $C_1 > C_3 = C_5$, in caprylate $C_1 > C_3 > C_5 = C_7$. The most likely explanation is that butyrate, first synthesized from two identical C_2 fragments, was diluted by nonisotopic butyrate and that acetate units were then added in succession, the methyl-C of these units being linked to the carboxyl-C of the fatty acid synthesized in

the preceding step [Popják *et al.* (39)]. It remains uncertain if and to what extent these findings can be extended to the synthesis of longer chain fatty acid and to tissues other than the mammary gland. Elongation of higher fatty acid by addition of two carbons to the carboxyl end has been shown to occur in several instances, and most recently in experiments in which much of the radioactivity fed to the rats as laurate-1-C^{14} or myristate-1-C^{14} was recovered in the C_{16} and C_{18} fatty acid fraction [Stevens & Chaikoff (40); Anker (41)]. However, a more or less uniform distribution of the isotope has been found generally in the long-chain fatty acid synthesized from labeled acetate *in vivo* and *in vitro*. In discussing such a discrepancy, Popják (36) suggests that the demonstration of a stepwise synthesis of short-chain fatty acids in goat milk has been possible because the pools of these acids in the mammary gland are probably very small and, in addition, nonisotopic butyrate must have been added from the blood, or formed in the gland from hydroxybutyrate, of which large amounts are present in the blood of ruminants. Indeed in nonruminant animals (rabbit) the milk fatty acids were uniformly labeled just as it had been found for the body and liver fatty acids.

By experiments on tissue slices incubated with C^{14}-acetate or tritium water or both, Balmain, Folley & Glascock (42) have shown again marked differences in the effects of glucose, insulin, and glycerol, added *in vitro* to the mammary gland from rat, or from sheep, respectively. It is suggested that in the mammary glands of nonruminants (as in most other animal tissues), the energy required for fatty acid synthesis is derived from glucose metabolism, whereas in ruminants where large amounts of lower fatty acids are absorbed from the rumen, a cellular adaptation has taken place and energy is obtained chiefly from oxidation of acetate. *In vivo* the glycerol required for the esterification of fatty acids is readily available, but in the isolated tissues glycerol formation may become a rate-limiting factor. Glucose is the direct precursor of glycerol since in the milk fat of a goat injected with acetate-1-C^{14}, 95 per cent of the isotope recovered from the glycerol moiety was in the 1 and 3 carbons. This finding is in line with the sequence of reactions: acetate-1-C^{14}→glucose-3,4-C^{14} units in glycogen→glycerol-1,3-C^{14} [Popják, Glascock & Folley (43)].

Microorganisms.—For the study of the biosynthesis of lipids, microorganisms have the advantage that a net formation occurs in a system which was lipid-free initially. In the production of fatty acids and sterols by the molds *Fusarium lini* and *F. lycopersici* acetate is utilized much more efficiently than glucose, possibly because, with the latter compound, alcoholic fermentation occurs together with lipid synthesis. Saturated fatty acids are formed first and then dehydrogenated by specific enzymes to oleic and linoleic acids. When the medium contains acetate, addition of various naphthoquinones has little or no effect. However, in the presence of glucose and naphthoquinones lipogenesis is depressed and pyruvate accumulates, suggesting interference with carboxylase activity and a consequent decrease in the formation of C_2 fragments from which fatty acids and sterols are syn-

thesized [Maselli & Nord (44); Coleman & Nord (45); Coleman, Cefola & Nord (46)].

Higher plants.—Just as in animals and microorganisms, acetate is utilized preferentially for the *in vitro* synthesis of long-chain fatty acids in plant tissues [Stumpf & Newcomb (46a)].

Glucide-C⇄Fatty Acid-C

The probable pathway for the conversion of glucides to fatty acids is the formation of C_2 fragments by oxidative decarboxylation of pyruvate. In the experiments of Popjâk & Hunter (47) rabbits were given either C^{14}-glucose or C^{14}-acetate together with aminobenzoic acid. In either case, the same values were found for the ratio of the specific activity of the liver fatty acids or cholesterol to the specific activity of the "acetyl" excreted as the acetylated amino derivative. Moreover, after administration of acetate-2-C^{14}, pyruvate-3-C^{14}, or glucose-1-C^{14} to lactating rabbits, octanoic acid was isolated from the mammary gland and milk. In all three types of experiments 95 per cent of the C^{14} was uniformly distributed among the even numbered carbons, indicating the following sequence: glucose-1-C^{14}→pyruvate-3-C^{14}→acetate-2-C^{14}→octanoate-2,4,6,8-C^{14}. Evidence that the reverse conversion of fatty acid-C to glucose-C also proceeds through C_2 units has been obtained in diabetic dogs fed palmitate labeled in either carbon-1 or carbon-6. In line with the assumption that the acetyls formed by oxidation were chiefly converted to C_3 units and these condensed to glucose, the glucose isolated from the urine was symmetrically labeled with 80 per cent of the radioactivity in carbons 3 and 4 after palmitate-1-C^{14}, and carbons 1, 2 and 5, 6 after palmitate-6-C^{14} [Abraham, Chaikoff & Hassid (48)]. In other experiments on liver slices from normal and diabetic rats large differences were found in the amounts of C^{14} incorporated into glucose from acetate-1-C^{14} or acetate-2-C^{14} respectively. To explain these differences, a scheme was postulated which obviously may be applied also to the incorporation of fatty acid-C into glucide-C. The scheme suggests that definite isotope patterns in the components of the tricarboxylic cycle result from the continuous introduction of labeled acetate into the cycle and from isotope dilution by unlabeled molecules entering the cycle [Strisower, Kohler & Chaikoff (49)].

Oxidation

Several aspects of this topic, especially the role of organized enzyme systems [Green (50)], the participation of CoA[2] [Lipmann, Jones & Black (51)], and the relationship of fatty acid oxidation to the tricarboxylic cycle [Breusch (52); Krebs (53)], have been discussed at the 2nd International Congress of Biochemistry in Paris. The oxidation of fatty acids by preparations from animal tissues and microorganisms has been reviewed also by Kennedy & Lehninger (53a).

Isolated tissues.—Beta-oxidation of straight-chain fatty acids from C_4 to C_{12} by soluble enzyme systems from liver [Drysdale (54); Drysdale &

Lardy (54a)] or heart [Mahler (54b)] has been reported. The system, obtained by water extraction of acetone powder from rat liver mitochondria, withstands centrifugation at 120,000×g, is comparatively stable, and requires ATP[2] or ADP, besides an electron acceptor (ferricyanide or 2,6-dichlorophenol indophenol).

Further evidence that, besides the liver, most other tissues oxidize fatty acids has been presented. Slices from rats which had previously received palmitate-1-C^{14}, produced $C^{14}O_2$ with a specific activity which was often higher than that of the tissue fatty acid. This finding may indicate the presence of a metabolically active fatty acid fraction which does not equilibrate rapidly with the bulk of the cell fatty acid [Volk, Millington & Weinhouse (55)]. Isolated rat diaphragm oxidizes octanoate [Hansen & Rutter (56)] as well as fatty acids of blood serum [Wertheimer & Ben-Tor (57)]. Lung tissue exhibits a low R. Q. and produces acetoacetate in larger amounts than liver slices under the same conditions [Pittoni & Rossi (58)]. Fatty acid oxidation by slices and cell substructures of plant cotyledons has been studied by Newcomb & Stumpf (58a).

2,3-dehydrogenation.—The introduction of a double bond between carbons 2 and 3 could be the initial step in the beta-oxidation of fatty acids. The anaerobic dehydrogenation of fatty acids, described years ago by Kuhn & Livada (59) and by Mazza (60), has been reinvestigated by Blakley (61). The enzyme occurs in liver mitochondria and is rapidly inactivated at 38°. It is not inhibited by CN^- or by malonate and does not require any known co-factors except ATP. With low concentrations of the substrates all saturated fatty acids from C_4 to C_{18} are dehydrogenated at about the same rate. 9,10-Undecylenate and oleate are also attacked, whereas, according to Mazza (60), 2,3-octadecenoate is not dehydrogenated. The system is clearly different from the enzyme which desaturates stearate to oleate [Lang (62)] and which does require coenzyme I and is inhibited by oleate. Under conditions similar to those described by Kennedy & Lehninger (63) for the aerobic oxidation of fatty acids, the oxidation of octanoate by liver mitochondria with methylene blue as hydrogen acceptor proceeds further, more citrate than acetoacetate being produced in anaerobiosis [Appel & Kiese (64)]. Formation of the 2,3 unsaturated acid occurs also in the oxidation of butyrate by *Aspergillus niger*. However, the existence of an alternative pathway, not involving crotonate, is indicated. Indeed, in the presence of cyanide, the unsaturated acid is not attacked, but in the cyanide-inhibited system, while crotonate accumulates, some hydroxybutyrate and acetoacetate is still formed from butyrate [Mukherjee (65)].

Role of CoA.[2]—Weinhouse (66) suggests that, if oxidation of the fatty acids is preceded by esterification with coenzyme A, enolization of the carbonyl group in the thioester linkage may occur in a manner similar to the enolization of ordinary esters in the presence of relatively high concentration of OH^-. This reaction (which in biological systems is perhaps promoted by the proximity of proton-attracting groups in the enzyme proteins) would ac-

tivate the β-carbon because of its proximity to the double bond, and a keto group will be introduced in that position. The successive step is postulated as a thiolytic split of the keto-ester by an additional molecule of CoA to give acetyl-CoA and the CoA derivative of the next lower fatty acid, in a manner similar to that suggested by Lynen, Reichert & Rueff (67) for the splitting of acetoacetyl-CoA. Thus a long-chain fatty acid could be completely degraded to C_2 fragments without the intermediate formation of free short-chain fatty acids.

Preliminary data on the fractionation of enzymes involved in the activation of acetate, acetoacetate, and butyrate indicate initial phosphorylation of CoA, followed by interaction of phosphoryl-CoA with the various substrates. Butyryl-CoA is acted upon by the fatty acid oxidase with DPN[2] as electron acceptor, and acetoacetyl-CoA is formed, probably through the crotonyl-CoA stage [Green *et al.* (68); Green (50)].

Since pantothenate is a component of coenzyme A, deficiency of pantothenate may result in a decreased oxidation of fatty acids. Indeed, oxidation of caproate or butyrate is markedly depressed in liver homogenates from weanling rats previously maintained on a pantothenic acid-deficient diet [Cheldelin *et al.* (69)].

Acetate oxidation.—In animal tissues tricarboxylic acid cycle probably represents the major pathway for the final oxidation of the C_2 fragments formed by beta-oxidation of the long-chain fatty acids. Failure of nonlabeled intermediates of the cycle to equilibrate isotopically with C^{14}-acetate in suspensions of living cells has been generally ascribed to permeability barriers. However, according to Krebs, Gurin & Eggleston (70), these barriers are largely overcome in rapidly cooled yeast cells. In this material acetate oxidation is not inhibited by malonate, and no isotope dilution of the $C^{14}O_2$ occurs after addition of succinate, fumarate, malate, ketoglutarate, glyoxylate, formate, or acetoacetate. It is suggested that in yeast, and also in other microorganisms, the tricarboxylic acid cycle supplies intermediates for organic syntheses rather than being instrumental in acetate oxidation. In view of the stability of acetyl-CoA in the cell, the following pathway is tentatively suggested: $CH_3 \cdot CO \cdot CoA \rightarrow CH_2OH \cdot CO \cdot CoA \rightarrow CHO \cdot CO \cdot CoA \rightarrow$

$$COOH \cdot CO \cdot CoA \rightarrow H \cdot CO \cdot CoA + CO_2.$$

Unsaturated Fatty Acids

Formation.—The available evidence suggests that saturated fatty acids first are synthesized and these then converted to the corresponding unsaturated acids by tissue dehydrogenases which may be specific for certain positions in the chain (especially the 9, 10 position). The isotope distribution in the C_{16} and C_{18} unsaturated fatty acid of rats injected with myristate-C^{14} (41) is in line with this interpretation. This probably applies also to the formation of certain minor components of milk fats, such as the 9,10 unsaturated fatty acids with 10 or 12 carbons. However, direct addition of C_2 unit at the carboxyl end of preformed oleic, linoleic, and linolenic acid could per-

haps best explain the positions of the double bonds in the various C_{20} and C_{22} fatty acids isolated from brain phospholipids by Klenk & Bongard (71).

Oxidation.—Preliminary conversion to the saturated acid or shift of the double bonds to the 2,3 position probably precedes the oxidation of unsaturated fatty acids. At any rate, the chief pathway for the biological degradation of oleic acid is markedly different from its oxidation by chemical reagents such as $KMnO_4$ (oleic acid→9,10-dihydroxystearic acid→azelaic acid plus nonylic semialdehyde). Additional evidence on this point was obtained by Bernhard & Gloor (72) who fed deuterium-labeled azelaic acid, alone or together with triolein, oleyl alcohol, or various anilide- and amide-derivatives of oleic acid. No isotope dilution of the azelaic acid recovered from the urine could be detected. With the last mentioned derivatives (in which blocking of the carboxyl was presumably interfering with beta-oxidation), small amounts of succinate were identified in the urine, suggesting that omega-oxidation is easier than direct oxidation of the 9, 10 double bond. On the other hand, hydroxyacids are known to be actively metabolized in animals [Artom, Gagliani & Ventura (73)]. It appears that azelaic acid is an important step in their degradation, since 9,10-dihydroxystearate (as well as the 9,10-diketo acid) caused marked decreases in the isotope concentration of the urinary azelaic acid.

Branched Chain Fatty Acids

Occurrence.—Branched fatty acids, previously reported only in microorganisms and in a few animal species, have now been found in butter, in beef-, and mutton-fat [Hansen & Shorland (74); Hansen, Shorland & Cooke (75)] and, in large amounts, in the secretion product of glandula uropygii (the "preen gland") which is largest in aquatic birds [Weitzel & Lennert (76)]. Racemic 2-methyl-butyrate is also an important metabolic product of *Ascaris lumbricoides* [Bueding & Yale (77); Moyle & Baldwin (78)]. Among the short-chain branched fatty acids which occur in the rumen of ruminant animals, Shazly (79) has identified 2-methyl-butyrate, isovalerate, and isobutyrate which probably arise from the microbic degradation of isoleucine, leucine, and valine. These products are easily metabolized by slices of the liver, kidneys, and rumen epithelium [Annison & Pennington (80)]. It seems likely that the 14-methyl- and the 15-methyl-hexadecanoic acids in butter fat (74, 75) are formed by successive addition of C_2 units to the carboxyl ends of these branched-chain C_4 and C_5 acids.

Oxidation.—Various 2-alkyl-substituted myristates and stearates prepared synthetically, were easily absorbed by dogs. The methyl derivatives were completely metabolized. After the ethyl-, and even more after the propyl and butyryl derivatives were given, the corresponding substituted adipic acids were isolated from the urine. Apparently little or no steric hindrance of the beta-oxidation of the main chain occurs by methyl substitution in carbon 2. However, when the lateral chain is longer, beta-oxidation is slowed down, and a notable portion of the fatty acid undergoes omega-

oxidation [Weitzel (81)]. It should be noted that this is the first evidence for omega-oxidation of fatty acids with a chain longer than C_{12}.

Saturated Hydrocarbons

Confirming and extending previous findings [Stetten (82)], Bernhard, Gloor & Scheitlin (83) administered to rats a series of hydrocarbons from C_2 to C_{18}, labeled with deuterium in the 1,2, or in the 2,3 positions. All compounds were readily absorbed and metabolized as indicated by the increased isotope content of body water. When octadecane was given, the stearic, oleic, and palmitic acids of liver and carcass fat were labeled, indicating oxidation of one methyl, and subsequent 9,10 desaturation or beta-oxidation of stearate. Fatty acids formed from shorter-chain hydrocarbons were not stored but caused an increase in isotope content of the volatile fatty acids of the urine.

PHOSPHOLIPID METABOLISM

Contributions on this topic are thoroughly covered in a monograph by Wittcoff (84). Other recent reviews deal with the synthesis of phospholipids in animal tissues [Artom (85)], with the mechanism of lipotropic factors in animals [Artom (86)] and in humans [Cayer (87)], and with the enzymes splitting phospholipids [Zeller (88)].

Synthesis

Intermediates.—The structural formulas of phospholipids suggest a number of possible intermediates many of which have been actually found in tissues. Most recently, glycerylphosphorylethanolamine was characterized [Campbell & Work (89)], and the widespread occurrence of phosphorylethanolamine was confirmed [Ansell & Dawson (90); Campbell & Work (89)]. By a simple procedure the amounts of glycerylphosphorylcholine in several mammalian tissues have been determined [Schmidt *et al.* (91)]. It is, of course, uncertain whether these and similar products represent intermediates in the biological synthesis of phospholipids, or in their hydrolysis, or in both. However, minced brain tissue synthesizes phosphorylethanolamine *in vitro*, and when P^{32}-phosphate was injected intracysternally into the brain of intact rats, the specific activity of phosphorylethanolamine phosphorus was many times greater than that of lipid phosphorus (90). Previous evidence for a role of glycerophosphate as a phospholipid precursor in intact animals and tissue slices is somewhat equivocal. However, in washed liver mitochondria, the uptake of P^{32}-phosphate by the phospholipids is markedly enhanced by addition of glycerol, and isotope dilution experiments with unlabeled glycerolphosphate indicate that the latter compound is involved in the process [Kennedy (92)]. Aqueous extracts of rat liver fractionated with methanol, convert P^{32}-glycerolphosphate into compounds which are probably phosphatidic acids. The conversion is accelerated by addition of long-chain fatty acids, of CoA[2] and, especially, of ATP. When the same preparations were

incubated with doubly labeled phosphorylcholine, approximately the same ratio P^{32} to C^{14} was found in the lipids synthesized as in the substrate [Kornberg & Pricer (93)]. The latter findings suggest that in a given tissue phospholipid synthesis may occur by alternative pathways, starting with either glycerolphosphate or phosphorylcholine.

Mechanism.—In intact animals differences in the rate of turnover of the phosphate and fatty acid [Weinman *et al.* (94)], or of the phosphate and choline moieties [Tolbert & Okey (95)] could not be detected. Probably under physiological conditions the constituents of the phospholipid molecule are not renewed at markedly different rates, and a relative independence in the turnover of these moieties becomes apparent only under conditions which affect specifically the synthesis of one or the other of the various ester linkages. Evidence on this point is supplied by a comparison of the results of experiments on the *in vitro* synthesis of phospholipids from various labeled substances, such as P^{32}-phosphate (Artom & Swanson (96)], C^{14}-choline [Artom, Crowder & Swanson (97)], or C^{14}-dimethylethanolamine (DME) [Crowder & Artom (98)]. In agreement with previous results on intact animals [Artom & Crowder (99)], the last compound is incorporated into the phospholipids of liver slices even before methylation to choline. Incorporation of phosphate into the lipids of liver slices is practically abolished by absence of O_2 or by homogenization of the tissue, whereas incorporation of choline or DME[2] proceeds readily in slices under nitrogen and is decreased, but not suppressed by homogenization. Liver slices from rats on low protein diets incorporate less phosphate but more choline or DME than slices from rats on stock diet. The uptake of choline or of DME by liver phospholipids is depressed by cyanide, azide, or dinitrophenol to a much lesser extent than phosphate incorporation. Presumably formation of the linkage between the nitrogenous component and the phosphatidic acid moiety has lower energetic requirements than synthesis of the phosphate-glycerol bond. Moreover, incorporation of choline or DME into the phospholipids of liver slices is inhibited by *in vitro* addition of ethanolamine, as well as of a number of precursors or analogues of ethanolamine and choline, suggesting that these various nitrogenous components compete with each other for the glycerolphosphate or for the phosphatidic acid radical. Such a competition might perhaps explain the rapid changes in the relative amounts and in the rate of formation of lecithins and cephalins which have been noted previously in the liver of intact animals receiving choline or ethanolamine or related substances.

Influence of diet.—As mentioned above, a low protein diet markedly depresses the *in vitro* synthesis of liver phospholipids from P^{32}-phosphate. Supplementation of the low protein diet with choline prevented the fatty infiltration but did not raise to normal the low rate of phospholipid synthesis (96). On the contrary, in the liver of intact rats on protein-deficient diets no decrease in the rate of phospholipid synthesis is detectable. Likewise, partial hepatectomy or toxic damage of the liver does not reduce the amounts of

phospholipids synthesized in a given time interval, the reduction in the number of cells or in their functional capacity being compensated by an increased rate of synthesis per unit of tissue (85, 86). Additional evidence on these points has been published recently [Campbell & Kosterlitz (100); Govaerts, Dallemagne & Gerebtzoff (101); Johnson & Albert (102); Singal *et al.* (103)]. It seems likely that regulatory mechanisms, possibly of hormonic nature, maintain phospholipid synthesis in the liver of intact animals at a level adequate to the metabolic requirements, such a level being much below the maximum functional capacity of the normal tissue. On the other hand, in the isolated tissue where regulating mechanisms are absent, phospholipids are formed at the highest rate compatible with the conditions of the experiment. The decrease in cytoplasmic materials, especially enzyme proteins, occurring in protein-depleted rats will then result in an actual decrease in the synthesis of phospholipids from inorganic phosphate.

Relation to fatty acid metabolism.—The finding that in certain tissues phospholipid turnover is accelerated under conditions in which the metabolism of fatty acid is presumably increased, may represent circumstantial evidence for a role of phospholipids in fat metabolism. Recent experiments on depancreatized or phlorizinized dogs in which there is extensive catabolism of fats, showed that phospholipid turnover was markedly increased in the liver, small intestine, and blood plasma, probably also in the kidney, but not in muscle and brain [Zilversmit & DiLuzio (104); DiLuzio & Zilversmit (105)]. These results almost exactly duplicate those obtained by increasing the fat content of the diet of normal rats [Artom, Sarzana & Segré (106)].

The increased phospholipid turnover after administration of choline or related compounds also suggests a role of lecithins in the lipotropic effect. However, several compounds enhance phospholipid turnover without being lipotropic [for a discussion of this point, see Cornatzer & Artom (107)]. A further example of such compounds would be triethylcholine which, contrary to previous claims, did not show any lipotropic activity under the conditions of experiments by Strength, Schaefer & Salmon (108). Moreover, the current interpretation that choline is lipotropic because it promotes mobilization of fatty acid as plasma phospholipids has become doubtful since several results, especially from Chaikoff's group, indicate that plasma phospholipids do not represent a major form for the transport of fatty acids. Preliminary data indicate that choline administration favors the oxidation of fatty acids in the liver. Indeed, production of $C^{14}O_2$ from stearate-1-C^{14} is markedly depressed in liver slices or homogenates from rats on choline-deficient diets. Choline administration to the deficient animals promptly restores the ability of the isolated tissue to oxidize the added fatty acid. Since *in vitro* addition of choline was not effective, the active substance is probably not choline but some substance formed *in vivo* from choline [Artom (81, 109)]. This substance was not identified; but, as a mere speculation, a role of lecithin for the activity of enzymes involved in fatty acid oxidation might

be suggested. Quastel & Braganca (110) have shown that the phospholipase A of snake venom does not affect soluble enzymes but inactivates all oxidative enzyme systems which are located in the mitochondria, indicating that the activity of such systems depends upon the intact phospholipid molecule. Lecithin appears also to be an integral part of certain enzymes, such as muscle adenosinetriphosphatase [Kielley & Meyerhof (111)], and large amounts of phospholipids have been found recently in a highly purified preparation of adenosinetriphosphatase from rat liver nuclei [Swanson & Mitchell (112)].

Dietary Fatty Liver

In a reinvestigation of the effects of cholesterol added in various amounts to choline-free, or choline-supplemented diets, it was found that at the lower cholesterol levels, the increases in both glycerides and cholesterol esters of the liver were prevented by choline, whereas at the higher levels of dietary cholesterol, choline was effective only against accumulation of fat [Ridout *et al.* (113)]. Analyses of the liver and carcass fat of rats on low protein diets with or without added choline, indicate that this substance affects the amount, but not the composition of fatty acids or their synthesis from glucides [Raman (114)]. Sellers & You (115) have extended their observations on the "pseudolipotropic effect" of a cold environment and of hyperthyroidism in rats on low-choline diets. It would appear that the increased expenditure of energy occurring under these conditions is made without choline participation, or that, if choline is involved, either the synthesis of choline in the body is increased or the limited amounts of available choline are utilized more efficiently. Additional evidence for a "choline-sparing effect" of cobalamin (vitamin B_{12}) is presented in a comprehensive report by Strength, Shaefer & Salmon (108). The results seem to be in line with others suggesting that cobalamin is involved in the synthesis of methionine, although probably by an indirect mechanism, such as the formation of *p*-aminobenzoic or folic acid [Dubnoff (116); Gibson & Woods (117); Stekol, Weiss & Weiss (118); Stekol *et al.* (119); Block, Stekol & Loosli (120); Liener & Schultze (121)]. Two recent papers indicate that carbon-2 of histidine is a major source of both methyl and ethanolamine carbons of choline. The incorporation of histidine carbon into choline is enhanced in choline-deficient animals [Sprinson & Rittenberg (122); Toporek, Miller & Bale (123)]. Additional evidence has been presented that "ceroid" and similar pigments found in the liver of choline-deficient rats, as well as in the tissues of vitamin E-deficient animals, result from the auto-oxidation of unsaturated fatty acid [Hartroft (124); Bruce Casselman (125)].

Hydrolytic Enzymes

There is increasing evidence for the widespread occurrence in tissues of a variety of specific enzymes acting on each of the ester linkages in the phospholipid molecule. The nomenclature of these phospholipases [see Belfanti, Contardi & Ercoli (126)] is indicated in the following scheme:

$$\text{choline} \xrightarrow{\text{(C)}} \text{phosphate} \xrightarrow{\text{(D)}} \text{glycerol} - \begin{cases} \text{(A) Fatty acid} \\ \text{(B) Fatty acid} \end{cases}$$

The formation of a complex between lecithin and the heat-stable phospholipase A of pancreatic extracts has been reported. The complex is soluble in ether where the reaction proceeds unimpaired. In agreement with previous findings only unsaturated fatty acids are released [Hanahan (127)]. However, Zeller (128) has obtained lysolecithin by the action of snake venoms on a fully saturated synthetic dimyristoyllecithin, and postulates that the decisive factor is the position of the ester linkage in the glycerol moiety rather than the unsaturation of the fatty acid. From a comparison of the splitting of lecithins and lysolecithins by fresh and aged extracts of acetone powders of the pancreas, Shapiro (129) suggests that, in addition to phospholipases A and B, the fresh preparations contain a new enzyme catalyzing the transfer of one fatty acid from lecithin to an acceptor which is present in the boiled tissue. Acker, Diemar & Jäger (130) have obtained stable preparations of the phospholipase C, first described by Hanahan & Chaikoff (131) in carrots. Activity of the enzyme increases with growth of the plant. A similar phospholipase is also present in takadiastase and in germinating wheat seeds.

CHOLESTEROL METABOLISM

Synthesis

Extrahepatic tissues.—In vitro experiments have demonstrated that many tissues besides the liver synthesize cholesterol from small molecules. In intact rabbit receiving labeled acetate the specific activity of cholesterol is often greater in the intestine than in the liver [Popják & Beeckmans (132)]. A similar relationship has been found in rats fed stearate-1-C^{14} [Borgström (133)]. Since in the latter experiments cholesterol is probably synthesized from the C_2 fragments produced by oxidation of the fatty acid, it is apparent that a very active metabolism of both fatty acids and cholesterol takes place in the intestinal mucosa.

Cell damage.—Liver homogenates, including the homogenate of pigeon liver which is quite active for synthesis of fatty acids (30), do not incorporate labeled acetate into cholesterol. However, occurrence of this process in homogenates of rat fetal liver has been reported [Rabinovitz & Greenberg (134)]. On the other hand, while complete disruption of the cell structure suppresses or greatly impairs the synthesis of cholesterol, experiments on pig liver perfused with blood containing C^{14}-acetate indicate that incorporation of this compound into cholesterol is enhanced by a less extensive damage of the organ such as that resulting from interruption of the circulation, mechanical trauma, or bacterial toxins [Werthessen & Schwenk (135)].

Pantothenate deficiency.—A previous report [Klein (136)] that yeast

grown in the absence of pantothenate synthesizes very little ergosterol, has been confirmed. By shifting the deficient microorganism to a medium containing pantothenate and C^{14}-acetate, ergosterol with a very high specific activity can be obtained [Hanahan & al-Wakil (137)]. In normal rats addition of cholesterol to an adequate diet causes fatty liver and high cholesterol levels in liver and serum. These changes are suppressed or reduced in pantothenate deficient animals, possibly as the result of a greatly reduced synthesis of cholesterol and fatty acids. It is suggested also that cortico-adrenal impairment in pantothenate deficiency might be due to a failure in the synthesis of a common precursor of ketosteroids and cholesterol from acetyl CoA[2] [Guehring, Hurley & Morgan (138)]. A drastic reduction of liver and plasma cholesterol esters was observed in pantothenate deficient rats, but this effect was reversed by adding fat to the diet [Boyd (139)].

"Fasted" and "diabetic" livers.—As for the incorporation of acetate into fatty acids, the synthesis of cholesterol from labeled acetate is markedly reduced in liver slices from rats previously fasted or maintained on a severely restricted diet. Oral administration of glucose or of proteins restored the ability of the tissue to synthesize both cholesterol and fatty acids. However, since the ingestion of fat, which is without effect on fatty acid synthesis, led to an increased formation of cholesterol, it would appear that the synthesis of cholesterol, unlike that of fatty acids, is not dependent directly upon the glycolytic activity of the cells [Tomkins & Chaikoff (140)]. A further instance of a condition which affects in different ways the synthesis of cholesterol and fatty acids is that of liver slices from diabetic rats. In these preparations incorporation of labeled acetate is higher than in liver slices from normal animals. Pretreatment of the rats with insulin reduces the rate of the process [Hotta & Chaikoff (141)]. These findings are of obvious interest in view of the hypercholesterolemia of diabetics and of the high incidence of arteriosclerosis in these patients. On the other hand, they are not inconsistent with the hypothesis that acetoacetate is an intermediate in the synthesis of cholesterol but not of fatty acids. It seems possible that in the isolated liver of diabetic animals formation of acetoacetate and therefore also of cholesterol are enhanced, as the result of a decreased utilization of active acetate for fatty acid synthesis. A similar interpretation can perhaps be suggested also for the increased synthesis of cholesterol in the partially damaged liver (135).

Intermediates.—In a study by Wuersch, Huang & Bloch (142) the isooctyl chain of cholesterol, synthesized by liver slices from either acetate-1-C^{14} or acetate-2-C^{14}, has been degraded. The results indicate that carbons 20, 23, and 25 are derived from acetate carboxyl and the remaining five carbons from acetate methyls. Such a distribution could be explained by the intermediate formation first of acetoacetate and then of C_5 units, according to the following scheme:

$$2\dot{C}H_3\cdot\overset{*}{C}OOH \rightarrow \dot{C}H_3\cdot\overset{*}{C}O\cdot\dot{C}H_2\cdot\overset{*}{C}OOH \rightarrow (\dot{C}H_3)_2\overset{*}{C}O + \overset{*}{C}O_2;$$

$$(\dot{C}H_3)_2\overset{*}{C}O + \dot{C}H_3\overset{*}{C}OOH \rightarrow (\dot{C}H_3)_2\cdot\overset{*}{C}:\dot{C}H\cdot\overset{*}{C}OOH \rightarrow (\dot{C}H_3\cdot)\dot{C}H_2:\overset{*}{C}\cdot\dot{C}H:\overset{*}{C}H_2$$

(where · identifies acetate CH_3 carbon and * identifies acetate COOH carbon). Condensation of a number of these isoprene units might yield squalene, a compound which has been suggested repeatedly as a precursor of cholesterol.

The presence of this branched polyunsaturated hydrocarbon in materials other than fish oils has been reported. It is apparently a major component of the unsaponifiable matter of human sebum [Sobel (143); MacKenna, Wheatley & Wormall (144)] and of the Vernix Caseosa of the fetus [Čmelik, Petrak-Longhino & Mihelie (145)]. It has been found also in dermoid cysts of the ovary [Dimter (146)], but not in skin atheromas [Čmelik (147)]. Evidence for the synthesis of squalene and for its conversion into cholesterol in mammals has now been presented [Langdon (148); Langdon & Bloch (148a)]. Squalene was not found in rat tissues, but when nonisotopic squalene was fed together with labeled acetate, the hydrocarbon containing C^{14} in significant concentrations could be recovered from liver and intestine. The labeled squalene, obtained by biosynthesis, was then given to mice. As much as 10 per cent of the isotope was recovered in the tissue cholesterol. The specific activity was much higher in cholesterol than in the fatty acids, indicating that conversion of squalene to cholesterol did not involve formation of C_2 intermediates.

Presumptive evidence for a precursor even closer to cholesterol was found by Schwenk & Werthessen (149) in experiments on isolated organs perfused with blood containing C^{14}-acetate. The digitonide obtained from the blood contained a highly radioactive substance, from which cholesterol could be separated only by conversion to the dibromoderivative.

Conversion to Other Products

Cholesterol administration causes marked increases in the 7-dehydrocholesterol of various tissues. Conversely, ingested 7-dehydrocholesterol is apparently hydrogenated to cholesterol. For these processes as for cholesterol synthesis, the intestinal mucosa seems to be especially active [Glover, Glover & Morton (150)]. Hydrogenation of the 4,5 double bond of 7-dehydrocholesterol might perhaps explain the formation of 7-cholestenol which constitutes as much as 30 per cent of the rat skin sterols (as compared with only 1 per cent of 7-dehydrocholesterol) [Moore & Baumann (151); Idler & Baumann (152)].

In the experiments mentioned above in which C^{14}-acetate was added to the blood circulating in the isolated liver, a compound with a specific activity lower than that of cholesterol, was isolated and identified as 3,5,6-trioxycholestane. It is obviously formed by oxidation of the newly synthesized cholesterol, but it remains uncertain whether it is produced by the surviving tissue or during the isolation procedure [Schwenk, Werthessen & Rosenkrantz (153)]. Conversion of cholesterol to cholic acid, previously demonstrated in dogs [Bloch, Berg & Rittenberg (154)], has been shown to occur also in rats. Tritium-labeled cholesterol was fed to rats with cannulated bile ducts, and isotopic cholic acid was isolated. The reaction is probably irrevers-

ible, since in rats receiving tritium-labeled cholate, the cholesterol from both tissue and blood was inactive [Byers & Biggs (155)].

Catabolism

The experiments of Chaikoff *et al.* (156) on intact animals and those of Meier, Siperstein & Chaikoff (157) on tissue slices indicate striking differences in the metabolic lability of the nucleus and of the lateral chain of cholesterol, respectively. Indeed, no isotopic CO_2 was produced from cholesterol labeled in carbon 4 of ring A, whereas carbon 26 was readily oxidized to CO_2. For the latter conversion, liver is the most active tissue and brain the least. Several microorganisms, especially when grown on cholesterol-containing media, oxidized cholesterol to 4-cholestenone [Arnaudi & Colla (158)]. In the experiments of Stadtman & Cherkes (159) C^{14}-cholesterol (prepared biosynthetically and therefore uniformly labeled) was oxidized completely to CO_2 by a *Mycobacterium* grown on cholesterol as the sole carbon source. Cells grown on glycerol produced only cholestenone: a finding which suggests that the further oxidation of this product involves an adaptive enzyme. Cell-free extracts from sterol grown cultures failed to produce CO_2, but converted cholesterol to a variety of products. Ketosteroids were also produced from cholestenone-4-C^{14}.

Cholesterolemia

Alfin-Slater *et al.* (160) found that cholesterol synthesis is not affected by the level of fat in the diet in rats which received D_2O. Previous findings [Gould & Taylor (161)] were confirmed that dietary cholesterol, while causing increases in blood and liver cholesterol, depresses cholesterol synthesis. Upon removal of cholesterol from the diet, return to normal values for both synthesis and cholesterol levels was accelerated by a fat-rich diet.

As for the effects of endocrine disturbances, a possible relationship of cholesterol synthesis to the diabetic hypercholesterolemia (140) has been mentioned. On the other hand, in the experiments of Byers *et al.* (162) on hyper- or hypothyroid rats receiving tritium water, the mean half-life of cholesterol in blood and tissues was 7 days in the hyperthyroid and 50 days in the hypothyroid rats, as compared with a value of 20 days in normal animals. Apparently thyroid hormone has opposite actions on cholesterol synthesis and on other factors upon which the level of blood cholesterol is also dependent.

Among these factors, accumulation of bile salts in blood seems to be the immediate cause of many hypercholesterolemias. Injection of sodium cholate increases the cholesterol level in the plasma of intact rats and enhances further the hypercholesterolemia following biliary obstruction or nephrectomy. Dehydrocholate or glycocholate are not effective [Friedman & Byers (163); Byers & Friedman (164)]. Increases in the blood levels of both cholate and cholesterol are found in hypothyroidism and in other experimental and clinical conditions [Friedman, Byers & Rosenman (165); Friedman & Byers (166)].

Bile Cholesterol

Simultaneous determinations in bile and blood plasma clearly indicate that the amounts of cholesterol in bile are largely independent from the cholesterolemic levels. On the other hand, in all conditions in which the synthesis of cholesterol is presumably increased (hyperthyroidism, young growing animals) or decreased (partial hepatectomy, toxic damage of the liver, thiouracil administration), the cholesterol content of bile varied accordingly. It seems, therefore, that biliary cholesterol is chiefly related to the synthesis of cholesterol in the liver rather than to its excretion as unchanged cholesterol [Byers & Friedman (167); Rosenman, Friedman & Byers (168); Friedman, Byers & Rosenman (169); Rosenman & Shibata (170)]. This statement is not inconsistent with the recent data of Siperstein & Chaikoff (171) who found that, after administration of cholesterol-4-C^{14}, the isotope was excreted chiefly via bile. Actually as much as 90 per cent of the C^{14} in bile was present as saponifiable compounds (presumably bile salts).

By a new procedure of fractional extraction, Isaksson (172) has shown that human bile contains large amounts of lecithin which is present in rather stable associations with bile salts. These associations may be important for the dispersion of cholesterol in bile. Polonovski & Bourrillon (173) also found that lecithin represents between 15 per cent and 20 per cent of the dry weight of the bile.

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METABOLISM OF AMINO ACIDS AND PROTEINS[1]

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The reviewer has been impressed by the difficulty of discussing the metabolism of the amino acids in the consecutive fashion that is possible with the carbohydrates, for example. The interruption of the discussion occurs after the amino acids have entered the blood stream from the intestine: here the discussion usually jumps to the many chemical alterations which may occur in the cells. We know much less about how the extracellular fluid is kept adequate as a nutrient medium for the cells with regard to amino acids than how it is kept suitable as far as carbohydrate nutrition is concerned. This subject has been considered here in close relation to that of protein synthesis. In spite of considerable preoccupation with the latter subject the more distinct advances of the year appear to lie in a third area, that of the biological synthesis and degradation of certain individual amino acids. None of these subjects has been considered completely because of space limitations.

Amino acid nutrition at the cellular level.—Every cell must have present in it, and presumably at certain limiting concentrations, a complete stock of amino acids in order to maintain the synthesis of protein and of other essential substances. This applies to nonessential as well as to essential amino acids. All the reactions which destroy amino acids or interconvert them must stop far short of depleting the supply of each and therefore must be sensitive to the supply.

The main factor which determines how the free amino acids are distributed in the animal organism is the activity of the cells in taking up amino acids, apparently against concentration gradients. Changes in the relative intensity of this "concentrative" activity are probably involved in changes in relative rates of growth and of destruction of amino acids (see discussion, Ref. 1). The transfer of amino acids from one extracellular fluid to another against gradients, e.g., absorption from the intestine, from the kidney tubule and from the maternal to the fetal circulation (2, 3) is probably a result of differences in the concentrative process at two surfaces of a cell.

The problem of providing every cell with adequate levels of each amino acid is well illustrated by some recent observations of Hokin (4). Ten amino acids were found to be stimulating to the synthesis of amylase by slices of pigeon pancreas, namely tryptophan, arginine, threonine, valine, leucine,

[1] The survey of the literature pertaining to this review was concluded in December, 1952.

[2] The reviewer was a Fellow of the John Simon Guggenheim Foundation during a portion of the time spent in preparing this review.

isoleucine, histidine, lysine, phenylalanine, and tyrosine. For this tissue and this synthesis, tyrosine became an "essential" amino acid, whereas methionine, not present in amylase, was without effect. But omitting each "essential" amino acid from the medium had only a moderate effect on amylase synthesis; undoubtedly a considerable part of the amino acid requirement was being met by proteolysis in the sliced tissue. In the intact animal this protein-secreting organ must be much more dependent upon the supply of free amino acids which it obtains from the extracellular fluid. Furthermore, the nonessentiality of methionine undoubtedly applies only to the synthesis of amylase and not to the total activities of the organ. In general, one would anticipate that those tissues which interconvert or synthesize amino acids more slowly would receive most of their amino acids, essential and nonessential, preformed from the blood stream, the interconversions occurring more rapidly elsewhere. A longer list of "essential" amino acids is more to be expected than a shorter one for a single cell or for a tissue, than for the whole animal. A reservation as to this conclusion comes from the work of Rafelson, Winzler & Pearson (5). Unexpected synthetic ability of the minced brain of the day-old mouse was found for various amino acids, essential and nonessential. Most of the amino acids came to be radioactive upon incubation with uniformly C^{14}-labeled glucose and the minced brain. Other tissues of the new-born mouse did not produce this effect, nor did the brain of the adult mouse (6). In agreement, Steele (7) found no labeling of six essential amino acids in a three- or four-months old mouse fed labeled sucrose. The results of Rafelson *et al.* do not yet show whether a net synthesis of essential amino acids occurs. For the intact rat, details on the tryptophan (8) and threonine (9) requirement have appeared, also on the isoleucine requirement of man (10).

Free amino acids as precursors of proteins.—A special importance of the concentrative activity of cells for amino acids undoubtedly is that it provides a reserve supply of precursors for protein synthesis. This is especially important because no other possible precursors representing the various amino acids have so far been detected, except in much lower supply. Dialyzable amino acid conjugates found in rat liver and muscle represented at the most about 2 mg. of nitrogen per 100 gm. of tissue (11) after omitting from consideration glutathione, carnosine, and anserine. A preparation of peptides of larger molecular size, insoluble in trichloroacetic acid, obtained earlier from liver by extraction with boiling water, represented 1 to 3.6 mg. of bound α-amino nitrogen per 100 gm. of liver [Borsook, *et al.* (12)]. This material has been found to include at least four substances [Fels & Tiselius (13)]. To assess the value of this material as reserve precursor we need to know whether its composition is responsive to the pattern of amino acids supplied. If one amino acid is absent, will the other amino acids be retained in the form of the precursors to permit later supplementation? The holding of precursors of protein in reserve in the cells is particularly important because it is very unlikely that the synthesis of a protein can be

completed if even a single amino acid is lacking. Mutual supplementation among amino acid mixtures not supplied at the same instant is no doubt possible because of the temporary increase of the cellular accumulations produced by the incoming amino acids. Amino acid conjugates would have small importance as reserves of precursor, even disregarding their inappreciable concentration, unless they showed similar responsivity.

The scarcity of intermediate precursors has been observed also in *Saccharomyces cerevisiae* by Halvorson & Spiegelman (14). They found that none of the amino acids were depleted from the cells when the utilization of one of them for growth or enzyme formation was prevented by the presence of a synthetic analogue. Therefore no appreciable quantity of intermediate precursors appeared to be involved in the synthesis of the enzyme or other proteins, except for possible precursors already so complex as to require the entrance of the amino acid whose utilization was blocked.

Imbalance among amino acids.—High levels of one or more amino acids can have the effect of preventing the cell from capturing other amino acids (15) or of utilizing them. This is one of the hazards inherent in the structural similarity of the amino acids. Many instances of antagonisms among amino acids have been reported this year, both in bacteria (16 to 22) and in higher animals (23 to 34). The principal defense of an organism against an excess of an amino acid is undoubtedly the accelerated degradation or conversion to other amino acids which results in most species from the elevated concentration. In certain enzyme deficiencies, for example in oligophrenia phenylpyruvica, the organism is unable to rid itself of the accumulated excess of an amino acid. The effect of such a persistent accumulation upon cell nutrition deserves attention.

Factors which might be expected to accelerate the degradation of amino acid repeatedly have been observed to suppress its antagonistic effect (cf. 24, 25, 26). Given an adequate degradative capacity no unbalancing effect may be demonstrable (cf. 15, 27). If a high level of one amino acid tended to inhibit the synthesis of another this would be expected to aggravate the imbalance; if, on the other hand, both its synthesis and utilization were inhibited the effects might cancel each other [Umbarger & Magasanik (20)]. The latter effect would tend to restore a balance between the two concentrations. For pairs of amino acids which are interconvertible the relation between their concentrations undoubtedly plays a large part in determining the net direction and extent of interconversion, so that the extracellular fluid can provide the relatively stable pablum that it does for the cells. In re-establishing a balance among the amino acids the organism is limited by the well-known restrictions of some of the interconversions in one direction or the other. Two studies have concerned the interchanges of nitrogen (35) and of carbon skeletons (36) of amino acids in the intact animal. Histidine, lysine, and threonine did not receive significant amounts of N^{15} from other amino acids. Interestingly, phenylalanine appeared to receive more N^{15} from fed tyrosine than from other labeled amino acids; likewise the leucines

appeared to receive more N^{15} from each other and from valine than from other labeled amino acids [Åqvist (35)].

An interesting case of antagonism among amino acids has been described by Kihara & Snell (22). In the absence of vitamin B_6 the amino acids glycine, D-alanine, and L-alanine had to be supplied to *Lactobacillus casei*. The L-alanine was supplied more successfully in the form of various small peptides than in the free form; these peptides apparently were better because they bypassed an interference by D-alanine or glycine with the uptake of L-alanine into the cells. The process of cellular amino acid capture was emphasized several years ago as a site of antagonisms among amino acids [Christensen, Streicher & Elbinger (15)]. The low specificity of the concentrative process, compared with most enzymic reactions (37) probably makes it especially subject to antagonisms, even among relatively dissimilar amino acids.

D-Amino acids have been involved prominently in the antagonisms among amino acids; slower disposal may be a factor. Their essential role in some microbial species [Camien (21); Kihara & Snell (22)] intensifies the iikelihood of antagonisms. Amino acid racemases have been dealt with in two papers (38, 39). The ability of the rat to use D-methionine and D-phenylalanine was decreased by the presence of other D-amino acids in the diet [Wretlind (28, 29)].

Why the reaction sequence from tryptophan to niacin should be particularly sensitive to imbalance has not been explained. Possibly an especially high cell level of tryptophan is required for these reactions, a level which cannot be maintained in the presence of excesses of other amino acids. Such a high level may be necessary to drive one of the reactions; or, instead, it may possibly serve to maintain an adequate level of the adaptive enzyme, tryptophan peroxidase (40). Another possible explanation is the inhibition of liver kynureninase by various amino acids which was noted by Goryachenkova (41). Threonine-induced imbalance in rats was avoided when free amino acids replaced casein in the diet (30, 31). Maddy & Swift (42) obtained slightly better utilization of amino acids in the rat when fed as casein than as free amino acids. The surprising feature was how slight the difference was, considering the inefficiency one might expect from the D-amino acids present and perhaps also from the excessively rapid absorption which occurs with free amino acids.

Proteins as possible precursors in protein synthesis.—With regard to transfer of amino acid nitrogen among cells, proteins have been supposed to play a large role, especially the plasma proteins. Results obtained by Barry (43) and Campbell & Work (44) indicate that the plasma proteins have no detectable part as precursors of milk protein. The work of Halvorson & Spiegelman (14) indicates that free amino acids rather than proteins were the precursors of an adaptively formed enzyme. Satisfactory evidence does not appear to be available for assigning a function of quantitative importance to the undegraded plasma proteins in transferring nitrogen from cell

to cell. McFarlane (45) has reviewed the use of isotopes in the study of plasma protein metabolism through 1951.

Yuile, *et al.* (46) produced plasma proteins labeled with lysine-ϵ-C^{14} in one dog, and fed them to a second dog. The fate of the isotopic carbon was essentially the same as when free labeled lysine was fed along with a protein digest, indicating that the homologous protein probably underwent digestion. In contrast, when the plasma protein was injected, as much as 20 per cent of the radioactive carbon was found after seven days in the circulating plasma protein and as much as 40 per cent in the tissues. The respiratory loss of labeled carbon dioxide was roughly only one-tenth as great as after feeding the proteins. This smaller oxidation of lysine was taken to imply that the lysine had not passed through the free state during the conversion of the injected proteins to tissue proteins. An alternative interpretation is that this lysine, released by the destruction of plasma protein so gradually and constantly 24 hr. a day, ought indeed to be used more extensively for protein synthesis than the fed lysine arriving in a large concentration over a short time. Furthermore, throughout the seven days the food lysine should continue to be much more liable to deamination than that coming constantly from the plasma proteins, so that the latter would be more likely to be reincorporated into protein.

A relevant question is, how sensitive are the turnover rates of the plasma proteins to factors which modify the rate of protein anabolism? Bartlett & Gaebler (47) after administering growth hormone to the dog have observed a more rapid turnover of some of the globulin fractions, also a more rapid synthesis of albumins, but without a matching destruction rate. Solomon & Tarver (48) found that a high protein intake accelerated the loss of methionine-S^{35} from the proteins of plasma, liver, and kidney. Hubay *et al.* (49) found no effect of protein depletion in the dog upon the rate of disappearance of albumin (labeled with a trace of I^{131}) from the plasma. When valine labeled with C^{13} in the isopropyl group was fed to rabbits during immunization with pneumococcal polysaccharide, the antibodies became more highly labeled than did the other plasma proteins [Gros *et al.* (50)]. The subsequent rate of disappearance of activity was rather similar among the various fractions. Forker & Chaikoff (51) have noted that the decreased rate of synthesis of muscle protein in diabetes is not associated with a decreased production of plasma proteins. Madden & Gould (52) found that the half-turnover time of the plasma fibrinogen was about four days in the normal dog, a period considerably longer than had been suggested by results with hepatectomized animals. Evidence has been obtained to indicate that the tobacco mosaic virus (53) and a bacteriophage (54, 55, 56) were built up from small molecules, part of them derived from the host protein, with ammonia playing a considerable role.

Peptides as precursors.—The very low level of tissue peptides, aside from specialized structures, has led to uncertainty as to the place of peptides as intermediates in the synthesis of protein. The simultaneous closure of all

the peptide links of a protein does not appear at all likely. Therefore, intermediates undoubtedly exist, although they may be transient and may not be released from the catalytic macromolecules. Their inappreciable accumulation suggests, in analogy to glycogen synthesis, that perhaps only one beginning is made on each polypeptide chain and that the reactions transforming the smaller intermediates are fast compared with those generating them (57). As far as transfer from cell to cell is concerned peptides serve very inefficiently, with sluggish entrance into cells (58), little if any absorption from the intestine (59, 60), and large urinary wastage upon injection (61). When glycylglycine or its amide were injected, they were apparently metabolized only after being split [Hoberman & Stone (62); cf. 63].

In supporting bacterial growth peptides usually have been found less active than free amino acids. Glycyl peptides served for the nutrition of *Leuconostoc mesenteroides* by way of hydrolytic cleavage [Virtanen & Nurmikko (64)]. In the few cases where peptides have proved more effective than amino acids the advantage has usually appeared upon closer examination to lie elsewhere than in the availability of the peptides for direct anabolic use. One interesting case of this kind has already been cited (22). In the same laboratory tyrosine peptides were found superior to free tyrosine, because the peptides released tyrosine gradually at low concentration, thus apparently decreasing the loss by decarboxylation [Kihara, Klatt & Snell (65)]. Valylglycylphenylalanine was observed to suppress the inhibition of valine uptake produced by other amino acids [Cohen, Hirsch & Reynaud (66, 67)]. Peptides of glycine and asparagine appeared to escape much of the deamination to which asparagine was subject [Miller & Waelsch (68, 69)].

The proteins of the egg of the sea urchin are known to redistribute themselves with great rapidity during the development of the egg. Glycine, alanine [Hultin (70)], and carbon dioxide (71) have been found to enter the proteins, especially the particulate proteins, during this process; this suggests that the redistribution occurs by way of free amino acids. Similarly the entrance of deuterium from heavy water of the medium, observed by Hoberman, Metz & Graff (72), was attributed to transaminations, reaminations, and enolizations,—reactions which are more likely to occur with free amino acids. Gustafson & Hjelte (73) have shown that substantial changes in the proportions of the various amino acids (combined plus free) occur during development, beginning mostly at the mesenchyme blastula stage or during gastrulation. The free glutamine and the nonprotein, nonamino acid nitrogen (spoken of as "peptides") increased during the early stages, before protein synthesis became rapid, and then decreased during rapid protein synthesis. Even if the unidentified nitrogen should prove to be due to peptides, it is not clear whether this material was intermediate in the breakdown or in the synthesis of protein.

All the evidence we have for the entrance of precursors other than amino acids into protein synthesis has been obtained indirectly. The entrance of

relatively large quantities of unknown protein precursors into ovalbumin synthesis was suggested by Anfinsen & Steinberg (74, 75) to explain a lack of uniformity in the labeling of aspartate or alanine at different points in the ovalbumin molecule. In contrast, no difference was noted by Muir, Neuberger & Perrone (76) in the specific activity of valine at terminal and nonterminal positions of hemoglobin formed in the intact rat supplied with labeled valine. It will be important to discover whether the terminal valines of globin represent several unique positions in polypeptide chains. In this case divergent specific activities could have been masked by their being averaged. Barry (43) concluded that the free lysine and tyrosine of the plasma could serve as the exclusive precursors of the lysine and tyrosine of casein in the goat. Campbell & Work (44), however, found a lower specific activity in the whey proteins than in the casein after feeding these two labeled amino acids to the rabbit. For the whey proteins a minor but significant participation by precursors other than the free amino acids appeared to be involved.

In the above experiments two sources of the hypothetical diluting precursor for the amino acid entering certain positions or certain proteins may be considered. First, any peptides synthesized from the free amino acids would themselves soon become labeled, and only those formed before the marked amino acid entered the cell would serve as diluent. In that case an increase in the length of the experiment should greatly diminish the divergence in labeling at various positions. Such an effect has been reported (75); a study of still shorter intervals seems desirable. Second, any intermediate breakdown products coming from the proteins of the system should continue indefinitely to be largely unlabeled. Possibly these can re-enter protein synthesis without complete breakdown. In this case the use of a minced tissue (74, 75) might be expected to intensify greatly the dilution, first because of the considerable proteolysis to be expected in such a system, second because of the smaller yield of new protein to be expected *in vitro*. The experiments *in vivo* (44, 75, 76) should be more suitable for determining the normal participation of various precursors. Much less dissymmetry of labeling of ovalbumin was observed *in vivo* (75). These interesting experimental approaches bring into prominence the question of the homogeneity of the loci of protein synthesis, especially the possibility of local amino acid gradients within the cell during rapid protein synthesis or proteolysis.

Considerable attention, as illustrated by a recent symposium (77), has been given to the possibility that peptides or amides can serve in protein synthesis in another way, by donating amino acids to unidentified acceptors. Hanes *et al.* (78) have shown that cabbage leaves contain an enzyme which catalyzes the transfer of glycine from various glycyl-peptides to various amino acids. The enzyme also hydrolyzes the original peptide, but this cleavage is inhibited by the presence of a suitable amino acid as an acceptor. This behavior is much like that of the proteolytic enzymes studied by Jones *et al.* (79) and Fruton (80). In the latter experiments peptides served also as acceptors of the amino acids, especially from amides, under the catalytic

action of proteolytic enzymes. Hendler & Greenberg (81) tested with negative results the possibility that glycine supplied as γ-glutamylglycine might enter protein more rapidly, or that the dipeptide might stimulate the uptake of amino acids into protein. Several whole-cell and particulate systems were studied. Free glycine was not observed to enter γ-glutamylglycine; furthermore, this peptide was rapidly split. Brenner *et al.* (82, 83) have observed the surprising formation of D-methionyl-D-methionine when the isopropyl ester of D-methionine was incubated with a liver homogenate. As far as optical specificity is concerned this activity appears to present a contrast with the over-all protein-synthetic reactions of mammalian tissues.

Even if peptides or amides should be found to function in protein synthesis as amino acid donors, it is another question whether exogenous peptides in general can serve as amino acid acceptors in the cell and thus be built up to proteins. Quite possibly when we supply an amino acid in the form of a dipeptide, we have already overrun the point at which it enters the biosynthetic reactions. It should soon be possible by controlled degradation procedures for proteins to determine whether or not a doubly-labeled peptide can serve as a direct precursor of the corresponding sequence of two amino acids in a protein.

A possible meaning of the extensive breakdown which usually appears to occur when one protein is converted to another is that the formation of free amino acids maintains other synthetic reactions which involve amino acids, for example, the synthesis of niacin, epinephrine, purines, or thyroxine. In any case it is clear that it is the free amino acids which are the chief precursors of synthetic as well as degradative reactions and whose distribution must be operative in determining the direction which nitrogen metabolism will take,—anabolic or catabolic.

Endocrine factors in amino acid distribution. Pathological changes.—If the distribution of free amino acids in an organism is largely determined by the activity by which cells capture amino acids, this activity should be considered as a possible site of endocrine effects upon amino acid distribution. Changes of the plasma and cellular amino acids should be in the opposite direction if this is the point affected. Observations with the pituitary growth hormone are consistent with the view that it stimulates the uptake of amino acids by some of the tissues. The hormone is known to lower the plasma amino acids, and recently Bartlett & Gaebler (84) noted that it increased the total amount of free amino acids which appeared to be contained in the dog. The presence of insulin may be necessary for this action: Wijnans & de Jongh (85) observed that the lowering of plasma amino acids was not obtained in eviscerated rats unless insulin was administered. Results from Luck's laboratory have indicated in turn the participation of other hormones in the lowering of plasma amino acids by insulin; the release of epinephrine appears to be necessary. Other sympathomimetic amines did not share the action of epinephrine [Brunish & Luck (86)]. Insulin has been reported to increase the incorporation of labeled DL-alanine into the protein of excised rat diaphragm,

provided that neither glucose nor pyruvate was present in the medium [Sinex *et al.* (87)]. As to the adrenal hormones, a prompt rise of cellular amino acids was reported after adrenal stimulation but not after cortisone administration [Lee & Williams (88)]; adrenocorticotropin was reported to decrease the uptake of histidine by both the renal tubular cells and extrarenal tissues [Grob (89)] and also to increase its oxidation and incorporation into tissue proteins [Novak (90)]. In Cushing's syndrome unusually rapid excretion of heavy nitrogen supplied in glycine has been reported [Parson *et al.* (92)]. A unified view for the effect of the adrenal steroids on amino acid distribution is not yet possible. The possibility that steroids may modify the extent to which substances are concentrated by cells is already well exemplified in connection with the distribution of inorganic electrolytes.

The increase of the plasma amino acids observed after heptatectomy has been found to be minimized by the injection of glucose [Flock *et al.* (93)]. Differences in the amounts of glucose used for maintenance probably account for the differences in the response of various amino acids observed in different laboratories (94, 95). The aminoaciduria of diabetes in dogs concerns most of the amino acids [Ivy *et al.* (96)]. Beaton, McGarrity & McHenry (97) have reported the plasma glutamate concentration to be elevated in malignant neoplasia. Patients with rheumatoid arthritis are reported by Lemon *et al.* (98) to show a greater fall in plasma glycine after the administration of sodium benzoate than do normal persons. Moore & Stein (99) have studied in detail the aminoaciduria of Wilson's disease and have confirmed the interesting increased copper excretion. Scheinberg & Gitlin (100) have reported pronounced decreases in the concentration of ceruloplasmin, a copper-containing plasma protein, in Wilson's disease. Dustin, Moore & Bigwood (101), and Childs (102) have emphasized the aminoaciduria in newborn infants.

One is encouraged to see a tendency to supplement paper chromatography with quantitative methods in the study of amino acid distribution. This is especially necessary in evaluating tissue-fluid relations. One notes an occasional tendency to restrict discussions of amino acid distribution to results obtained by one or another group of methods. The reporting of amino acid concentrations in weight terms, traditional for the microbiological assays, is gradually being replaced by terms (e.g., millimolarity) which permit a ready summation and comparison of levels. At least one polemic, besides much unnecessary calculation, has had its origin in the weight terms.

Comparison among species.—Detailed analyses have emphasized the astonishing cellular amino acid levels of many marine invertebrates [Camien *et al.* (103); Gustafson & Hjelte (73); cf. 104] and have shown high levels in yeast [Lindan & Work (105)], in marine (106) and nitrogen-fixing algae (107), and in a protozoan [Wu & Hogg (108)]. In crustaceans the free amino acid composition of the nerves (109) appears to differ from that of the muscles (103) by the substantial excess of anionic over cationic amino acids in the former. Studies have continued on the strong amino acid accumulation by

bacteria [Gale & Van Halteren (110)]. Preliminary studies have indicated a similar activity in tubercle bacilli (111). In connection with these high amino acid levels in lower animals the possibility has been considered that during specialization restraint is imposed upon a primitive activity, to the advantage of efficiency in the transfer of amino acids among cells (37). Presumably, the more tightly a single-celled organism retains its amino acids the better, but in a multicellular organism excessive concentration would leave the extracellular levels too low for efficient exchange among the cells. The loss of concentrative activity of rabbit reticulocytes (112) upon maturation [coincident with a loss of incorporative activity for leucine into protein (113, 114, 115)], also the high amino acid levels of fetal and regenerating tissues and of neoplastic cells (1, 116), would be in harmony with such a concept. This restraint may be of endocrine nature or under endocrine control.

Nature of the concentrative process.—In contrast to the concentration of glycine by the rat diaphragm (117), and the accumulation of glutamate by cerebral slices [Stern *et al.* (118)] and by bacterial cells [Gale (119)], the uptake of several amino acids by erythrocytes, nucleated and nonnucleated, has been shown not to require oxygen gas and not to be readily inhibited by cyanide or dinitrophenol (120); the process, however, is highly temperature sensitive. Presumably in these cells glycolysis serves to provide energy. In Ehrlich ascites carcinoma cells an intense amino acid concentration was shown (1, 121); this involved over 20 amino acids, including D-isomers and unnatural structures (37). Observations of Boulanger and his associates (122, 123), of Campbell & Work (124) and of Heath *et al.* (125) indicate that β-alanine, taurine, and ergothioneine are probably also included among the substances concentrated by cells. In the Ehrlich carcinoma cells the process of concentration was readily inhibited by oxygen exclusion and respiratory inhibitors.

Two types of antagonism among pairs of amino acids for uptake by *Staphylococcus aureus* have been observed by Gale & Van Halteren (110), one of which apparently involves extracellular formation of a peptide by the two acids. A similar process could not be detected in the ascites-tumor cell; in fact three peptides interfered with the uptake of the amino acids contained in the peptides (58). In the carcinoma cell the antagonisms among amino acids were limited to amino acids of like charge [cf. Kamin & Handler (126, 127)]. Fluoroacetic acid appeared to be an inhibitor of amino acid concentration by cells (Awapara, 128).

α,γ-Diaminobutyric acid was concentrated so strongly by the carcinoma cell that most of the cellular potassium was replaced by the organic cation; a progressive uptake of water, amino acid, and chloride produced intense swelling (129). The organic cation was observed to be a stronger competitor for concentration by the cell than potassium. Perhaps the temperature-dependent lysis of *E. coli* cells in glycine solutions is a related phenomenon [Gordon *et al.* (130)]. When these organisms were trained to grow in the presence of glycine they also became resistant to the lytic effects of other amino

acids, but were then lysed by water unless salt was added (131). In the tumor-bearing mouse the liver rather than the tumor cell captured most of the diaminobutyrate, until about half of the liver potassium had been traded for the organic cation (129). The curious behavior of diaminobutyrate was partly shared by α,β-diaminopropionate.

Diamino acids other than those found in protein are turning up with astonishing frequency in peptide antibiotics, including one more occurrence each for α,γ-diaminobutyrate (132) and diaminopropionate (133) and three occurrences of a new amino acid, isolysine (134, 135, 136). α,ϵ-Diamino-β-hydroxypimelic acid has been found in the toxin of *Pseudomonas tabaci* [Wooley *et al.* (137)]. Meso-diaminosuccinic acid has been observed to interfere with the growth and nitrogen assimilation of a number of microorganisms [Suzuki *et al.* (138)].

The subjects of amino acid and potassium concentration by the cell have drawn close together, the relation not being limited to charged amino acids. Elevated potassium levels inhibit the uptake of glycine whereas elevated glycine concentrations cause the loss of potassium from the cell, with sodium replacement (121). Amino acids taken up to high concentrations by carcinoma cells appear to retain their full osmotic activity and electrical charge (121, 129). This does not establish that these amino acids are free in the cell, but if they are bound, it must be to a binding agent present in at least 0.1 *M* concentration, and in a combination in which the osmotic activity of the amino acids is preserved. The situation may be different in *S. aureus*, where swelling appears not to have been recorded during the formation of large gradients. Of course, the possibility always needs to be kept in mind that the "free" amino acids of the cells may represent labile intermediates in protein synthesis.

The amino acid "pool."—The supply of a given amino acid which is in the free state in the organism consists of numerous cellular "pools" communicating by way of the extracellular fluid. In each tissue the amino acid will have different rates of exchange with the extracellular fluid and with its precursors and products in the cell. Administration of a labeled amino acid, therefore, will lead to a different specific activity in each compartment. Arnstein & Neuberger (139; cf. review, 140) have shown that the amount of glycine available for hippuric acid synthesis varied with the dose of benzoate injected. A large dose increased the interval during which conjugation continued. This permitted secondary glycine pools outside of the viscera to enter into the reaction, and also permitted greater dilution in the liver of the labeled glycine by the reactions which replace the glycine with new glycine. After an injection of labeled glycine, its rapid capture by the liver led to very high initial specific activities there, which were probably never equalled in such organs as muscle. This inhomogeneity of the amino acid "pools" must be taken into account in trying to measure indirectly either the amount or specific activity of an amino acid in a given tissue at a given time.

Isotopic methods.—An important paper by Brunish & Luck (141) indi-

cates that amino acids can become firmly bound to proteins without enzyme participation. They found that preparations of liver desoxyribosenucleohistone and histone could "incorporate" labeled amino acids upon incubation with them *in vitro*, the incorporation being insignificant at room temperature, faster at 37° than at 42–45°, but much faster still at 70° or 100°. The amino acids fixed at 100° met several of the tests for "true" incorporation, the most impressive being the stability to ninhydrin at 100°. Parallel studies of the stability for the amino acids bound at 37° were not reported. The processes at the two temperatures may not have been the same, considering that there were two temperature optima. This is a property which deserves full study. The quantities of isotopic amino acid used by Brunish & Luck were relatively very large and the fraction taken up very small; furthermore the active material represented only a small fraction of the liver. The findings do not appear to justify wholesale rejections of earlier results; nevertheless they show the need for great caution, especially in connection with broken-cell preparations showing relatively low incorporation. At the present time the only unequivocal test for incorporation appears to be the isolation of labeled small peptides after partial degradation of the protein.

A frontal attack on protein synthesis has been attempted in a study by Siekevitz (142). Mitochondrial and microsomal fractions were found to act synergistically, in the presence of an oxidizable substrate, to incorporate labeled DL-alanine into protein. The finding that dinitrophenol and the hexokinase system were inhibitory indicated the necessity of phosphorylation for alanine incorporation. A soluble factor, not an activated form of alanine, appeared to be formed when α-ketoglutarate and cofactors were incubated with the mitochondria. This factor stimulated alanine incorporation by the microsomal fraction.

Peterson & Greenberg (143) have studied extensively the incorporation of C^{14} added in the form of glycine to rat liver homogenates. Unfortunately, a very large part of the observed binding appears to have occurred by disulfide rather than peptide bonds. With labeled threonine one-third to two-thirds of the activity was removed by treatment with mercaptoethanol; with valine one-fifth was removed [Kit & Greenberg (144)].

Borsook *et al.* (113) have detected in plasma a nonprotein fraction which accelerated the incorporation of amino acids into proteins by reticulocytes. Results of Brachet & Chantrenne (145) with bisected giant-cell algae indicated, in agreement with Siekevitz and earlier work, that the nucleus is not necessary for protein synthesis. Brunish & Luck (146) observed in the intact rat a slower turnover of nuclear than of cytoplasmic protein of the liver. The results of Åqvist (147) emphasize the similarity in protein turnover in cytoplasm and nuclei during hepatic regeneration. Eliasson *et al.* (148) noted a correlation between the respective rates of incorporation of glycine into protein and into the purines of nucleic acids during liver regeneration, whereas Holmes & Lee (149) found that irradiation of the Jensen sarcoma severely inhibited nucleic acid synthesis, without having any consistent effect upon the incorporation of methionine-S^{35} into protein.

The uncertainty as to whether amino acids can enter preformed proteins by simple exchange reactions continues to hamper the interpretation of results. Gerarde, Jones & Winnick (150; cf. 151) found the incorporation of labeled amino acids into protein in cultured tissues to be more rapid under conditions of autolysis than under conditions favorable to a net increase of protein or of growth. Robertson (152) observed, in guinea pigs, an appreciable turnover of the glycine of collagen which was undiminished in scurvy; the labeled proteins were not, however, believed to represent newly synthesized molecules. Observations upon "procollagen," including the uptake of labeled amino acids, were consistent with the view that this material served as a precursor of part of the collagen (153, 154).

Transamination.—The broad biological scope of transamination has been re-emphasized (156, 157, 158). If an hour of preincubation was allowed after addition of the coenzyme, pyridoxamine phosphate and pyridoxal phosphate were equally effective in activating pig-heart apotransaminase [Meister, Sober & Peterson (159)]. Meister (160) has shown that enzymic transamination between L-isoleucine or L-alloisoleucine, on one hand, and α-ketoglutarate, on the other, involves no change in the configuration about the β-carbon. A similar configurational stability was observed during enzymic oxidation of the isomeric L-β-phenylserines to mandelic acid [Fones (161)]. *L. arabinosus,* in contrast to other species, used L-alloisoleucine for growth, the inversion to isoleucine probably occurring at the stage of the keto acid formed by transamination (160). Evidence that β-2-thienyl-DL-alanine underwent transamination in the presence of a liver mitochondrial preparation was reported (162). Transamination of oxidation products of cysteine will be discussed below (p. 248).

The distinctive, simultaneous transamination-deamidation reaction shown by glutamine with various keto acids has also been observed with asparagine, but a different enzyme system is involved [Meister *et al.* (163)]. Why these two changes of the structure of the amides of the dicarboxylic amino acids occur together is not yet understood. Appreciable deamidation occurred only in the presence of α-keto acids; inhibitors blocked both transamination and deamidation. Deamidation was probably not an initial step since the preparations were much less effective upon glutamate and aspartate than upon the amides.

Microbial synthesis of amino acids.—The pathways by which amino acids are synthesized by microorganisms have attracted great interest. In addition to the study of particular steps by blocked mutants (see below) another approach has involved the culturing of organisms on appropriately labeled carbon compounds (e.g., acetate, bicarbonate), followed by the isolation and degradation of amino acids to determine the position of the isotope. These studies have indicated the nature of the condensations by which a number of carbon structures are built; for example, they have thrown light on the photosynthetic reactions in *Rhodospirillum rubrum* [Cutinelli, Ehrensvärd *et al.* (164, 165)]. A number of the metabolic relations already known in other species have been observed. Gilvarg & Bloch (166) found that acetate

could serve as a precursor of most of the amino acids in yeast, but not of glycine, phenylalanine, or tyrosine. In yeast, alanine was not an important precursor of serine, whereas in *R. rubrum* alanine and serine were very similarly labeled. When the latter species grew on acetate and bicarbonate as carbon sources, 40 per cent of the protein carbon was derived from the methyl group of acetate, 22 and 12 per cent, respectively, from the carboxyl group and from bicarbonate (164). Similar studies were made on *E. coli* by Cutinelli *et al.* (167).

Abelson, Bolton & Aldous (168, 169) have shown the possibility of determining whether or not an amino acid was synthesized by *E. coli* partially from CO_2, by noting whether or not addition of the amino acid to the culture medium diminished the fixation of labeled CO_2. In *Brucella abortus* the fixation of CO_2 involved chiefly a surprising exchange of the carboxyl group of glycine with CO_2, which apparently occurred without loss or exchange of the amino group [Marr & Wilson (170)].

Citrulline, arginine, and ornithine.—The carbon chain of arginine, synthesized by *Torulopsis utilis* from various labeled substrates, showed a distribution of isotopic carbon consistent with a synthesis from α-ketoglutarate [Strassman & Weinhouse (171)]. Extensive conversion of glutamate to proline and arginine has been shown in the intact rat [Sallach, Koeppe & Rose (172)]. According to a preliminary report, N-α-acetyl-L-ornithine has been isolated and identified as a precursor of ornithine in *E. coli* [Vogel (173)].

Study of the intermediate formed by the reaction of carbamyl glutamate CO_2, ammonia, and adenosinetriphosphate in liver has continued [Grisolia & Cohen (174)]. At least two enzymes appear to participate in its formation. The intermediate, in turn, reacts with ornithine to form citrulline and to regenerate carbamylglutamate.

The cleavage of arginine by certain bacterial species has been shown to proceed via ammonia and citrulline and then to ornithine and CO_2; an extract which catalyzes the first stage in *Clostridium perfringens* was prepared [Schmidt, Logan & Tytell (175); cf. Oginsky & Gehrig (176), Slade & Slamp (177)]. The same reaction was observed in *Chlorella* by Walker (178), but if fumarate was added a compound taken to be arginosuccinic acid was formed. This is the intermediate which Ratner postulated in the synthesis of arginine from citrulline and aspartate. Other organisms apparently catalyzed the same reaction (179). Virtanen & Miettinen (180) observed citrulline to be the most abundant amino acid in nitrogen-fixing alders, the concentrations being highest near the nodules. A nitrogen-storing function was suggested for citrulline; asparagine and glutamine were absent.

Lysine.—α-Aminoadipic acid has been shown to be a precursor of lysine in *Neurospora* [Windsor (181)] but not in *E. coli* where diaminopimelic acid is a precursor [Davis (182)]. Dewey & Work (183) demonstrated in *E. coli* the presence of a decarboxylase which converts diaminopimelic acid to lysine. The enzyme was absent from three of the lysine-requiring mutants of Davis. Diaminopimelic acid has been found present in extracts and hydrolysates of various organisms, including *Vibrio cholerae* (184), *E. coli* (182), and

mycobacteria (185), but not in *Neurospora*. The existence of two distinct pathways for the biosynthesis of an amino acid is very unusual. Davis (182) suggested that the diaminopimelic pathway is followed only in organisms which require it as a structural component. Threonine is also related to lysine synthesis in *E. coli:* a mutant which responded only slowly to lysine and diaminopimelic acid accumulated threonine. Still another mutant grew rapidly on threonine and slowly on the other two amino acids. A partially reversible formation of all three from a common precursor was proposed (182).

Boulanger & Osteux (186) noted that the oxidation of lysine by an L-diamino acid dehydrogenase of fowl liver yielded ammonia but no keto acid. Cyclization of the primary oxidation product was proposed. Evidence for a cyclic product from lysine oxidation by the L-amino acid oxidase of *Neurospora* has also appeared in a preliminary report [Lowy *et al.* (187)].

Leucine, isoleucine, and valine.—Umbarger & Adelberg (188) have observed that the keto acids (as well as the dihydroxy acids) corresponding to isoleucine and valine supported the growth of several bacterial mutants requiring these amino acids. Former divergent results were caused by a misidentification of the supposed "ketoisoleucine." The isotope distribution found for acetoacetate and carbon dioxide derived from doubly-labeled 2-methylbutyrate in liver slices indicated that β-oxidation occurred on the longer chain of 2-methylbutyrate. The behavior of this substance supported its intermediary role in the degradation of isoleucine [Coon & Abrahamson (189)]. The C^{13} supplied to rats as L-valine labeled in the β-position appeared in the liver glycogen, especially in the 1,6- and 2,5-positions of the contained glucose [Peterson, Fones & White (190)]. The intermediate formation of a 3-carbon acid was indicated. Tumor-bearing animals showed a reduced capacity to convert valine to glycogen [Fones & White (191)].

The sulfur amino acids.—The study of blocked mutants of microorganisms has shown reaction sequences among the sulfur compounds which are similar to those in higher animals, in addition to special abilities to reduce sulfate and to incorporate sulfur into the amino acids. In *Salmonella typhimurium* two mutants were observed which required, alternatively, cysteine or methionine, indicating mutual interconvertibility of these two acids [Plough *et al.* (192)]. In *E. coli* a mutant required not only methionine but also another form of sulfur; labeled methionine failed to yield any other form of sulfur [Cowie & Bolton (193)]. Similarly, *Pasteurella pestis* appeared unable to convert methionine to cysteine [Englesberg (194)]. *Tetrahymena geleii* failed to convert homocysteine to methionine, even when a methyl donor and cobalamin (vitamin B_{12}) were supplied [Genghof (195)]. The requirement for methionine was therefore considered absolute. Cystine, homocystine, and other cysteine precursors could spare methionine, indicating that methionine could serve as a source of cysteine. Only 72 per cent of the labeled sulfate taken up by *Torula utilis* was accounted for as methionine, cystine, humin, and sulfate (196).

Upon heating to 100° with pyridoxal and metal salts, serine and cysteine

have been shown to be deaminated to yield pyruvate and ammonia, and also hydrogen sulfide in the case of cysteine [Metzler & Snell (197)]. Pyridoxal phosphate had already been implicated in the enzymic desulfhydration of cysteine (cf. 198, 199); pyridoxal phosphate was shown essential also for D-serine dehydrases prepared from *Neurospora* [Yanofsky (200, 201); Reissig (202)] and from *E. coli* [Metzler & Snell (203)]. A similar but distinct activity for homoserine in rat liver has been studied by Azarkh & Gladkova (199). The coenzyme apparently also functions in the formation and cleavage of cystathionine [Binkley *et al.* (203a); Goryachenkova (204)]. A review by Snell (205) on the properties of pyridoxal phosphate emphasizes the close correlation between its chemical behavior and the catalytic roles of enzymes which contain it, namely in decarboxylation, transamination, racemization [cf. (206)], as well as in serine dehydration and cysteine desulfhydration.

Mackenzie & duVigneaud (207) have observed that the extent to which the methyl group of methionine is destroyed by oxidation in the rat on a choline-free diet is greatly increased when choline is added, but again decreased when cystine is also included. The finding of Elwyn, Weissbach & Sprinson (208) that both β-hydrogen atoms accompany the β-carbon of serine in its conversion to the methyl groups of choline in the intact rat has imposed unexpected limitations on possible explanations of the transfer of this one-carbon unit. Further discussion of one-carbon metabolism has been omitted for lack of space.

Cysteinesulfinic acid was shown by Bergeret, Chatagner & Fromageot (209, 210) to be degraded by two enzyme systems in the livers of higher animals: by a desulfinicase which removes sulfur dioxide from the alanine portion of the molecule, and by a decarboxylase which releases CO_2 and hypotaurine. Intravenous injections of cysteinesulfinic acid led to increases in both the alanine and hypotaurine of the liver. Two different alternative pathways exist in cell-free extracts of *Proteus vulgaris*, according to Kearney & Singer (211). The cysteinesulfinic acid may transaminate directly with α-ketoglutarate, and the resulting β-sulfinylpyruvate be desulfinicated to pyruvate and sulfur dioxide, or, alternatively, the amino acid may be oxidized first to cysteic acid and then transaminated with α-ketoglutarate to yield β-sulfonylpyruvate. The latter transamination was also reported by Darling (212). Quantitative oxidation to cysteic acid occurred when transamination was blocked by the addition of semicarbazide, provided that an unidentified new coenzyme, "coenzyme III," from yeast was added (213). Cysteic acid formation could be blocked by adding a pyrophosphatase to destroy "coenzyme III."

The toxic effects of methionine sulfoximine, both upon dogs and *Lactobacillus mesenteroides*, have been earlier shown to be reversed by methionine. Pace & McDermott (214) now have found that the sulfoximine also interferes both with the glutamine-synthesizing enzyme system of sheep brain and with the glutamyl transferase of Schou *et al.*, especially with the latter. Only the L-isomer appears to be effective, and reversal was not obtained with methionine. The incorporation of S^{35}-labeled methionine into liver slices was

sharply reduced by the presence of methionine sulfoximine [Roth *et al.* (215)]. When S^{35}-methionine sulfoximine was administered, radioactivity was at first concentrated in the kidneys. but more persistently in the liver and intestine. Little was captured by the brain and spinal cord (214).

Proline biosynthesis.—The reaction sequence, glutamic acid→glutamic-γ-semialdehyde→Δ^1-pyrroline-5-carboxylic acid→proline, has been indicated for *E. coli* by Vogel & Davis (216). This sequence is the reverse of that for which Taggart & Krakauer secured evidence in mitochondrial preparations of rabbit kidney (217). The unsaturated cyclic acid was shown to satisfy the proline requirement in one blocked mutant and to be identical with a substance accumulated in another. During the attempted preparation of the γ-semialdehyde of glutamic acid by the acid hydrolysis of γ,γ-dicarbethoxy-γ-acetamidobutyraldehyde, spontaneous ring closure to Δ^1-pyrroline-5-carboxylic acid occurred. Davis has summarized his studies of amino acid biosynthesis in blocked mutants (218). As was noted earlier, in yeast the carbon chains of phenylalanine and tyrosine arose from glucose (166). The glucose passed through the triose stage, but not to pyruvate, before the synthesis of the skeleton of the amino acids (219).

Histidine.—During the early forties Japanese workers obtained strong evidence for the conversion of histidine to glutamate in liver by way of urocanic acid, formylisoglutamine, and glutamine (bibliography in reference 220). Sera (221) has reported the isolation and identification of DL-formylisoglutamine and DL-glutamine as intermediates and has separated and studied the two enzymes leading respectively to urocanic acid and to enol-formylisoglutamine. The first enzyme, "histidine α-deaminase," from liver, has also been studied by Hall (222). Tabor & Hayaishi (223) and Tabor *et al.* (220) have established clearly this pathway of histidine catabolism for extracts of *Pseudomonas fluorescens* showing that the α- and γ-N atoms appeared in the forms required by the scheme. Abrams & Borsook [(224); cf. 225)] have obtained confirmatory evidence for the importance of this pathway in rat liver, in the formation of a labeled glutamate precursor from carboxyl-labeled histidine, and in the presence of the label in other than the α-carboxyl position of the resulting glutamate. A reaction sequence passing through the ureide of glutamic acid rather than formylisoglutamine has also been proposed (226). Additional observations of the position of formate as both a precursor and a degradation product of carbon -2 of the imidazole ring have been reported (227 to 230). This carbon atom, fed as histidine to the rat, appeared in creatine and choline [Reid *et al.* (227); Toporek *et al.* (229)], although this does not mean that histidine should be considered a methyl donor, nor a lipotropic agent [Eckstein (231)]. Williams & Krehl (232) have shown that an increase in bound histidine, presumably representing carnosine, occurred when rat liver slices were incubated with histidine and β-alanine.

Microbial synthesis of the aromatic amino acids.—The reaction sequence, 5-dehydroquinic acid (233)→5-dehydroshikimic acid→shikimic acid (218, 234) has been detected by Davis *et al.* in the synthesis of the aromatic com-

pounds tyrosine, phenylalanine, tryptophan, *p*-aminobenzoic acid and *p*-hydroxybenzoic acid, by *E. coli.* Priorities for synthesis of these five aromatic metabolites appear to increase in the order listed. In certain mutants which accumulated dehydroshikimic acid because of a genetic block, shikimic acid met the requirements for only the last three of these five. This finding appears to be explained by an inhibition of shikimic acid utilization by the accumulated dehydroshikimic acid. The priority relations among the three aromatic amino acids may be due to the sequence in which they are synthesized (235). α-Phenylglycine has also been suggested as an intermediate, judging from its accumulation in a *Neurospora* mutant (236).

The enzyme tryptophan desmolase of *Neurospora*, which catalyzes the coupling of serine and indole has been studied by Yanofsky (237). A stimulating effect of niacin on the growth of mutants requiring tryptophan has been attributed to an accessory or catalytic action of niacin rather than to its conversion to tryptophan [Partridge *et al.* (238); cf. Beadle *et al.* (239)]. The metabolic relations of anthranilic acid, indole, and tryptophan appear to be quite different in *Lactobacillus arabinosus* (240). The synthesis of tryptophan in the rumen of cattle has been shown (241). Mutants of the mealmoth are able to synthesize tryptophan, the genetic block being in the conversion of tryptophan to kynurenine (242). Formaldehyde was released by the action of a partially purified demethylase on α-N-methyltryptophan [Tung & Cohen (243)].

Degradation of phenylalanine and tyrosine.—A soluble system which catalyzes the conversion of phenylalanine to tyrosine was extracted from rat liver by Udenfriend & Cooper (244). Sealock *et al.* (245), in a study of the action of ascorbic acid and similar compounds on the conversion of *p*-hydroxyphenylpyruvate to homogentisate, have obtained results which are largely in agreement with those of Knox & LeMay-Knox (246). Information is still lacking on the details of the pathway. The subject of alcaptonuria has been reviewed by Galdston, Steele & Dobriner (247). Roka (248) has confirmed the suggestion of Knox & LeMay-Knox (246) that the formation of alanine, noted in earlier experiments at Frankfurt, was a result of transamination rather than of a direct cleavage of the carbon skeleton of tyrosine. Kirkberger & Bücher (249) noted the excretion of *p*-hydroxphenylacetate by rabbits fed *p*-hydroxyphenylpyruvate or large amounts of tyrosine. Administration of 500 mg. of ascorbic acid tended to decrease the excretion of *p*-hydroxyphenylacetate. This substance has also been observed as a product of tyrosine degradation in kidney slices [Roka (248)]. Charconnet-Harding & Dalgliesh (250) noted that rats made pyridoxine-deficient by feeding sulfamezathine and desoxypyridoxine did not excrete administered tyrosine, as might have been expected if transamination had been stopped completely and if no alternate pathway existed.

The scurvy-alleviating action of cortisone did not include direct effects upon the excretion of *p*-hydroxyphenylpyruvate in the guinea pig [Basinski *et al.* (251)]. Administration of adrenocorticotropin usually decreased the keto acid excretion, but only transiently. In scorbutic monkeys, in contrast, the

administration of cortisone or adrenocorticotropin prevented excretion of *p*-hydroxyphenylpyruvate [Salmon & May (252)]. A number of analogues of the aromatic amino acids have been studied (162, 253 to 260).

Tryptophan degradation.—Figure 1 is drawn as a guide to the discussion, but very few of the steps are adequately verified and alternative pathways are not excluded. A valuable review by Dalgliesh has appeared (261). Steps 1 to 3 are the best studied (262, 263), but intermediate A remains unidentified. It is not oxindolalanine, the conversion of this substance to kynurenine being spontaneous and artefactual (264, 265). Steps 1 to 3 have now been confirmed for trytophan-adapted *Pseudomonas* [Stanier *et al.* (266)].

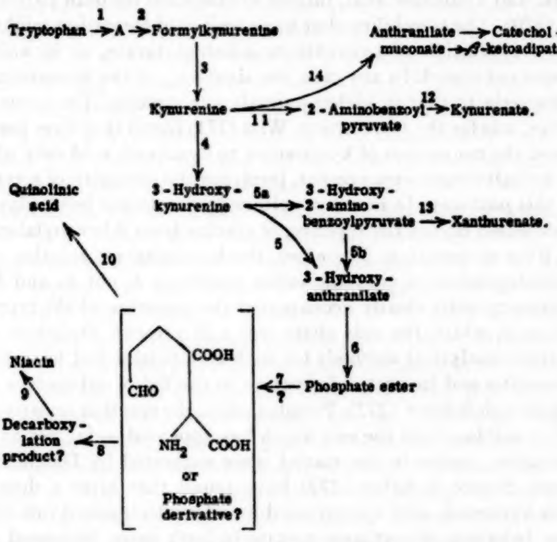

FIG. 1. Degradation of Tryptophan.

The greatest discussion concerns kynureninase action (step 14 or 5) and its relation to the alternative pathways: ring closure to kynurenic and xanthurenic acids (steps 12 and 13). Last year Dalgliesh, Knox & Neuberger (264) proposed that alanine release, under the action of kynureninase, occurred by a cyclic transamination in which pyridoxal reversibly accepts the amino group to yield a 2-aminobenzoylpyruvate; this α, γ-diketo acid is then split, the pyruvate again receiving the amino group from the coenzyme. The cyclization of the 2-aminobenzoylpyruvate to kynurenic or xanthurenic acids was regarded as a spontaneous event, intensified whenever the cleavage of the benzoylpyruvate derivative was inhibited. Pyruvate and α-ketoglutarate were noted to inhibit kynureninase action, presumably by accepting amino groups from the coenzyme. If the foregoing view is correct, the branching of the pathways of tryptophan degradation occurs at the stage of 2-amino-

benzoylpyruvate and 3-hydroxy-2-aminobenzoylpyruvate, and both pathways should exist in organisms where kynureninase is present. The rapid spontaneous cyclization of 2-aminobenzoylpyruvate and the consequent unavailability of this compound have greatly complicated its investigation. Hayaishi & Stanier (267) interpreted the action of the kynureninase of *Pseudomonas fluorescens* as yielding anthranilic acid and alanine directly, without such a cyclic transamination. Stanier *et al.* (266 to 270) have reported furthermore that every one of 24 adapted strains of *P. fluorescens* which could degrade tryptophan extensively did so by way either of anthranilic acid or of kynurenic acid, but not by both routes. Only one strain yielded both anthranilic and kynurenic acid, further degradation by both pathways being blocked (270). The possibility that kynurenic acid formation might be stimulated by the addition of pyruvate or α-ketoglutarate, as in animal preparations, was not tested. In any case, the similarity of the kynureninase action of the bacteria to that in higher animals was striking. The bacterial enzyme, however, was far the more active. Wiss (271) found that liver preparations catalyzed the conversion of kynurenine to kynurenic acid only when pyruvate or α-ketoglutarate were present, implying the necessity of a transamination for this pathway. In contrast neither pyruvate nor benzoylpyruvate could be detected during the splitting of alanine from β-benzoylalanine by a different liver preparation. Therefore, the kynureninase reaction was believed to be independent of transamination (pathway 5, not 5a and 5b). Pyridoxine deficiency quite clearly accentuated the excretion of the tryptophan metabolites in which the side chain was still present. Dalgliesh has studied extensively analytical methods for such metabolites and has added 3-hydroxykynurenine and its acetyl derivative to the list of substances observed in pyridoxine deficiency (272). Paradoxically, the reaction sensitive to pyridoxine deficiency has been the one which has appeared so far not to involve transamination, unless in the special sense suggested by Dalgliesh *et al.* (264). Porter, Stoerk & Silber (273) have found that after a dose of tryptophan, less kynurenic acid was excreted by adrenalectomized rats than by normal rats. Injection of cortisone acetate in both cases, increased the excretion of kynurenic acid. Porter *et al.* consider that the absence of cortical steroids interferes with the deamination step (no. 11) of kynurenic acid formation, diverting the metabolism of tryptophan toward side chain removal (step 5). Elucidation of the mode of action of cortisone by determining the activity of the various enzyme systems after cortisone administration will be desirable. Kynureninase from liver also split 3,4-dihydroxykynurenine to form alanine and 3,4-dihydroxyanthranilic acid; dihydroxykynurenine, however, did not promote the growth of niacin-requiring *Neurospora* mutants, nor was it a precursor of *Drosophila* eye-pigment (274).

Turning to the stages below anthranilic and 3-hydroxyanthranilic acids, anthranilic acid has been shown to be degraded to β-ketoadipic acid in *Pseudomonas* (266), probably with catechol and *cis, cis*-muconic acid as intermediates, since after adaptation of the bacteria these two compounds also were converted to β-ketoadipic acid. 3,4-Dihydroxyanthranilic acid was

probably not an intermediate between 3-hydroxyanthranilic acid and nicotinic acid, since it did not replace the latter for the nutrition of a *Neurospora* mutant [Hellman & Wiss (275); cf. 276]. The failure of this plausible intermediate to serve may be explained by the observation of Hellman (277) of the presence in liver preparations of a compound composed of 3-hydroxyanthranilic acid esterified with phosphoric acid at the hydroxyl group; perhaps oxidation in the 4-position occurs only after the phosphorylation. The possible part played by this phosphate group in the ring opening and closing, and the stage at which it is removed, remain as fascinating problems.

Schayer & Henderson (278) have shown by feeding experiments that the nitrogen of the indole ring of tryptophan is the source, and probably the only source, of the nitrogen of quinolinic acid, and therefore of nicotinic acid. This finding is consistent with the previously postulated opening of the benzenoid ring between carbons 3 and 4 (step 7). Quinolinic acid feeding led to the excretion of methylated derivatives of nicotinic acid in man [Sarett (279)], but L-tryptophan proved to be a considerably more effective precursor. This observation does not yet show us whether quinolinic acid belongs in the main pathway of niacin synthesis.

An additional degradation product of kynurenine, kynurine, has been observed in the pupae of *Bombyx* [Butenandt *et al.* (280)]. This substance could arise by a decarboxylation of kynurenic acid, or the decarboxylation could precede the ring closure. D-Tryptophan was about three-quarters as effective as L-tryptophan in the nutrition of the rat [Oesterling & Rose (8)]. The utilization of the D-isomer probably occurred by inversion via indolepyruvate [Mason & Berg (281)]. The species differences in tryptophan metabolism of the cat have been further described (282). Braunshtein *et al.* (283) showed that of the species studied only cats excrete anthranilic acid after tryptophan feeding. DaSilva, Fried & DeAngelis (282) have now found the cat unable to convert tryptophan into niacin. Further evidence for the conversion of tryptophan to niacin in the developing egg has appeared (284). L-Trytophan has been variously estimated as from three-tenths [Duncan & Sarett (285)] to fully as effective [Feigelson, Williams & Elvehjem (286)] as nicotinic acid, on a molecular basis, in stimulating the biosynthesis of pyridine nucleotides, the comparative efficiencies being modified by the dosage levels.

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THE CHEMISTRY OF CORTISONE[1,2]

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Introduction

The chemical investigation of cortical concentrates by independent groups of workers headed by Kendall, Kuizenga, Reichstein, and Wintersteiner, led to the isolation of 28 crystalline compounds in addition to cholesterol. Thus cortisone was isolated and described in 1936 by Mason, Myers & Kendall (178, 179), by Reichstein (231), by Wintersteiner & Pfiffner (308), and in 1939 by Kuizenga & Cartland (138); cortisol was isolated and described in 1937 by Reichstein (232), in 1938 by Wintersteiner & Pfiffner (308), and in 1939 by Kuizenga & Cartland (138). The structure of cortisone was elucidated by Reichstein (231, 232), whose formula was later adopted by Kendall *et al.* (180).

Although some 90 per cent of the original activity of the concentrates remains in the mother liquors as the "amorphous fraction," six (cf. Fig. 1) of the 28 compounds mentioned above were shown to be active in maintaining life in adrenalectomised animals: deoxycorticosterone, corticosterone, dehydrocorticosterone, Reichstein's substance S, cortisol, and cortisone. It soon became clear from the results obtained in methods of bioassay for other than life-maintenance that there was a marked qualitative difference between deoxycorticosterone and Reichstein's substance S, possessing no oxygen atom at $C_{(11)}$, and the other four compounds corticosterone, dehydrocorticosterone, cortisol, and cortisone all of which possess an oxygen atom at $C_{(11)}$. No amount of the former two compounds would produce the effects typical of the latter four compounds on carbohydrate and protein metabolism (increased levels of carbohydrate in the liver and the blood with simultaneous increased breakdown of protein to provide the carbohydrate and increased excretion of urinary nonprotein nitrogen). There were hopes that these four compounds might be of value for substitution therapy in Addisonian cases, and that in view of the bioassay methods of Selye (272, 273, 274) and Swingle (288, 289), they might be of value also for treatment of surgical and wound shock. There were also rumours in 1940, later proved to be false, that such cortical compounds were being used in Germany to enable pilots to operate without oxygen apparatus at great altitudes and to improve their resistance in black-outs from dive-bombing. In 1941 to 1942 a widely planned attack on the problem of the partial synthesis of such 11-oxygenated compounds, in particular DHCOS[2] and cortisone, was initiated in various American laboratories with the financial support of the United States Government. Owing to the wartime interruption of the circulation of scientific journals,

[1] The survey of the literature pertaining to this review was concluded in October, 1952.

[2] The following abbreviation is used: DHCOS for dehydrocorticosterone.

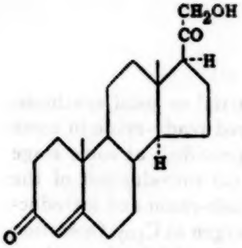
the American workers were unaware that Reichstein & Lardon (144) had synthesised 30 mg. DHCOS in 1942 to 1943 (see page 273), and much work was needlessly duplicated. However, in 1945, the Merck organisation prepared a large sample of several hundred grams of DHCOS by a method devised by Kendall (see page 273). In Addisonian patients DHCOS was found to be of little value, and there was no evidence to suggest that cortisone would be of any greater clinical interest or utility. In 1946 Sarett (262) synthesised 29 mg. of cortisone (see page 280) and after some improvements in the synthesis (see page 283), the Merck organisation in April 1948, produced a few grams of cortisone. There is little information as to the effect of cortisone on shock and trauma, although there is evidence that in small daily doses (10 to 20 mg.) it is a valuable addition to the therapy of Addison's disease. The principal claim to fame of cortisone lies in the dramatic effects produced by its administration in cases of acute rheumatic fever and chronic rheumatoid arthritis as described in 1949 by Hench *et al.* (100). Only cortisol (and adrenocorticotropin) appears also to be effective,[3] and there has been a tremendous demand for cortisone and cortisol for clinical purposes.

Only two naturally occurring steroids have been proved to contain an oxygen atom at $C_{(11)}$ (Figure 1). These are (*a*) the cardiac aglycone, sarmentogenin, which has been degraded to 3,11-diketoaetiocholanic acid [Katz (129)] and should be capable of conversion by ozonolysis to the appropriate $3\beta,11\alpha$,21-trihydroxy-20-ketone (194), and (*b*) the toad poison, gamabufogenin, which has been degraded to the same 3,11-diketoaetiocholanic acid [Meyer (196)]. Gamabufogenin has been isolated from the skins of Japanese and Chinese toads (*Bufo formosus*, *B. japonicus*, and *B. gargarizans*) and from the oriental drug Ch'an Su. A feverish search has failed to disclose a source containing more than $\sim$0.45 per cent of sarmentocymarin ($\equiv$sarmentogenin-sarmentoside) although many varieties of *Strophanthus* have been examined (71, 72, 249, 260); recently, however, seeds of the savannah form of *S. sarmentosus* have been reported to contain 0.86 per cent of sarmentoside-B acetate [$\equiv$sarmentogenin-digitaloside-glucoside hexaacetate], together with 0.27 per cent of sarmentogenin (after hydrolysis) (35, 36, 73). It seems improbable that either of these rare substances could be obtained or cultivated on the requisite scale as materials for the partial synthesis of cortisone.

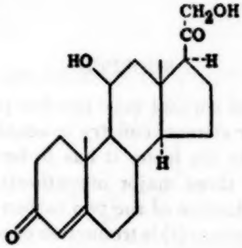
Passing reference should perhaps be made to tetracyclic triterpenes as a potential source for the preparation of cortisone analogues. Lanastadienol [$\equiv$lanosterol] from wool fat is one of the most abundant; the stereochemistry corresponds to that of the steroids[4] and the 9(10)-double bond is ideally suited to the introduction of oxygen at $C_{(11)}$; Ruzicka *et al.* (292) have shown that ring A can be transformed with elimination of the 1,1-dimethyl group to give a 6-membered $\alpha\beta$-unsaturated ketone, which gives in principle 14α-methylcortisone (Figure 1).

[3] It has been reported (238) that the 21-aldehydes corresponding to cortisone and cortisol have approximately the same activity in rat liver glycogen deposition tests.

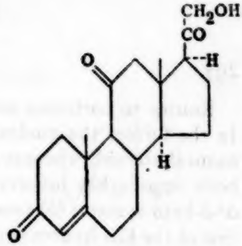
[4] Private communication by Dr. D. H. R. Barton.



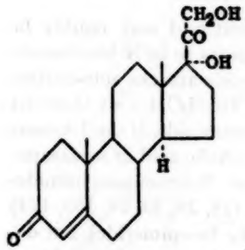
Deoxycorticosterone (DOC)



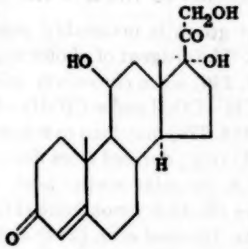
Corticosterone (COS)



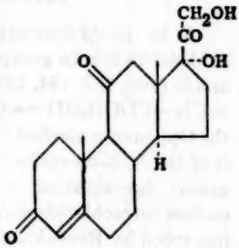
Dehydrocorticosterone (DHCOS)



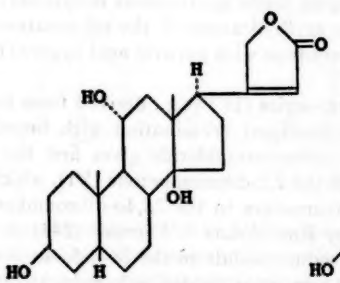
Reichstein's Substance S



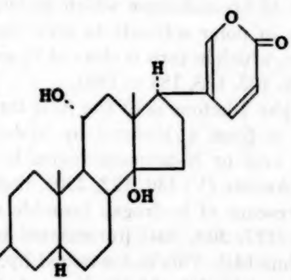
Cortisol
Kendall's Compound F
Reichstein's Substance M



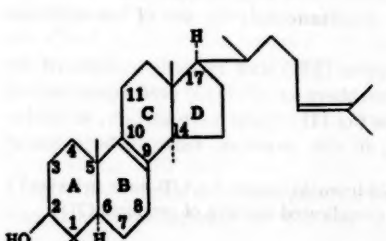
Cortisone
Kendall's Compound E
Reichstein's Substance F



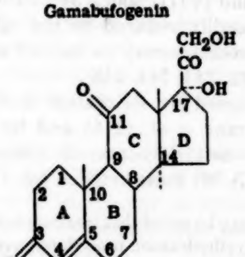
Sarmentogenin



Gamabufogenin



Lanastadienol



14α-Methylcortisone

FIG. 1.

Routes to cortisone and cortisol may involve partial or total synthesis. In the former the nuclear stereochemistry is acquired ready-made in some natural steroid, whereas in the latter it has to be provided; at some stage both approaches involve three major operations: (*a*) introduction of the Δ^4-3-keto-system; (*b*) production of the two carbon side-chain and introduction of the 17α-hydroxyl group; (*c*) introduction of oxygen at $C_{(11)}$. Operation (*b*) may precede or may follow operation (*c*); operation (*a*) is always last. Procedures which have been described for these operations will now be reviewed.

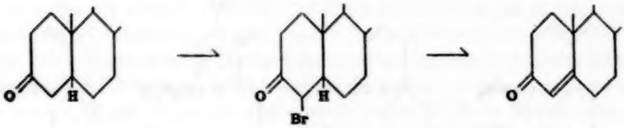
INTRODUCTION OF THE Δ^4-3-KETO SYSTEM

A 3α- or 3β-hydroxyl group is invariably present and may readily be oxidised to a keto group. The reagent of choice appears to be N-bromoacetamide (106, 132, 134, 137, 230) since chromium trioxide attacks side-chains: $=CH—COCH_2OH \rightarrow =CH—CO_2H$ and $=C(OH)—COCH_2OH \rightarrow =CO$, whilst the Oppenauer method (214, 229) may lead to D-homosteroids. If the 3-ketone is of the A/B-*cis*-series (I) (e.g., derived from deoxycholic acid or sarsasapogenin) bromination with bromine-acetic acid or N-bromosuccinimide-carbon tetrachloride gives the 4β-bromoketone (II) (28, 29, 30, 48, 105, 122) [regarded by Rosenkrantz, Djerassi *et al.* (244) as the 4α-epimeride], but dehydrobromination with hot pyridine or collidine affords reasonable yields of the $\alpha\beta$-unsaturated ketone (III) only if the $C_{(17)}$ side-chain is of simple type (133). Good results are however obtained by formation of the arylhydrazone of the 4β-bromoketone which undergoes facile spontaneous dehydrobromination in polar solvents to give the arylhydrazone of the $\alpha\beta$-unsaturated ketone, which in turn is cleaved by exchange with pyruvic acid to give (III)[5] (11, 48, 103, 133, 184 to 188).

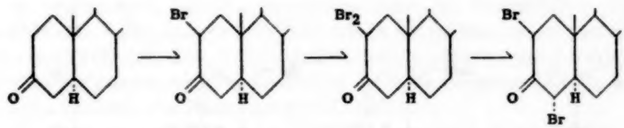
If the 3-ketone is of the A/B-*trans*-series (IV) (e.g., derived from hecogenin or from a Δ^5-sterol by hydrogenation) bromination with bromine-acetic acid or N-bromosuccinimide-carbontetrachloride gives first the 2β-bromoketone (V) (30, 122, 250), then the 2,2-dibromoketone (VI), which in the presence of hydrogen bromide rearranges to the 2β,4α-dibromoketone (VII) (122, 303, 304) [formulated by Rosenkranz & Djerassi (244) as the 4β-epimeride]. This is converted by sodium iodide to the 2α-iodo-4α-bromo compound (VIII) which by extended treatment yields the 2α-iodo-Δ^4-ketone (IX), readily reduced to the $\alpha\beta$-unsaturated ketone (III); alternatively, these processes may be carried out simultaneously by use of hot collidine (157, 242, 243, 244, 248).

As noted by Reichstein & Shoppee (235) and recently confirmed by Rosenkranz *et al.* (216) and by Hershberg *et al.* (213) hydrogenation of 11-keto- or 11β-hydroxy-Δ^4-3-ketones (as III)) yield very largely, or exclusively, A/B: *trans*-ketones (as IV); it has, however, recently been found

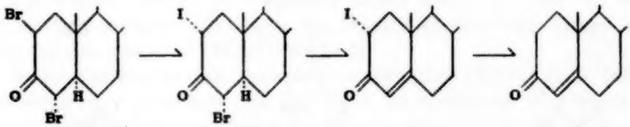
[5] It may be noted that treatment of a 2β-bromoketone of the A/B-*trans* series (as V) by the arylhydrazone procedure gave a complicated mixture of products (213).



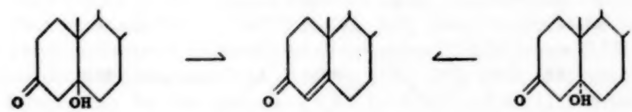
I II III



IV V VI VII



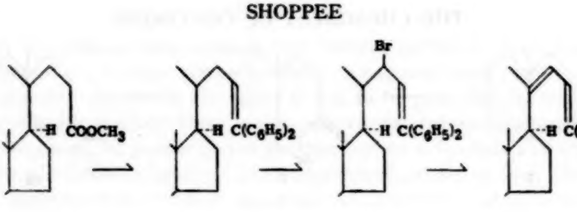
VII VIII IX X



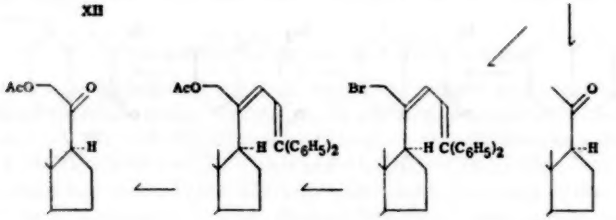
XI III XI

that hydrogenation of 11α-hydroxyprogesterone with palladium in an alkaline medium gives mainly 11α-hydroxypregnane-3,20-dione (as I) (156); this observation may be compared with that of Marker *et al.* (163), and with the more recent observation of Rosenkranz & Djerassi that hydrogenation of 3-keto-$\Delta^{4,7}$-dienes and 3-keto-$\Delta^{4,6,8(9)}$-trienes of the 22-isospirostan series also afford A/B:*cis*-ketones (as I) (54, 311), thereby allowing use to be made of the facile pathway (I→II→III) for reintroduction of the biologically important $\alpha\beta$-unsaturated keto-grouping.

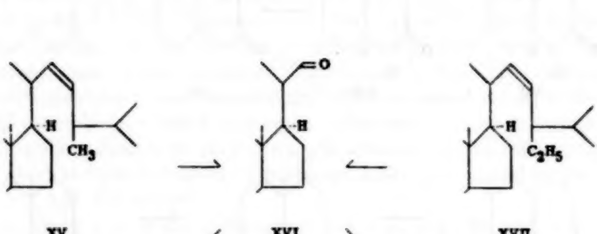
3-Keto-5-hydroxy steroids (XI) very easily furnish Δ^4-3-ketones (III) by treatment with acid (57 to 60, 74, 120, 147, 220), alkali (94, 123), or by pyrolysis [110°/0.01 mm.] (223); a 3-keto-5α-hydroxy compound derived initially from dehydroergosterol peroxide has recently been converted into 11-ketoprogesterone in this way (21, 22, 99, 121).



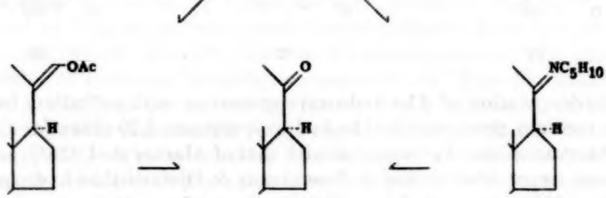
XII



XIV XIII



XV XVI XVII



XVIII XIII XIX

Production of the two Carbon Side-Chain and Introduction of the 17α-Hydroxyl Group

The two-carbon side chains of choice are: (*a*) $=CH—COCH_3$, and (*b*) $=CH—COCH_2OH$. Side chain (a) can be transformed into (*b*) by use of lead tetra-acetate (61, 69, 92, 155, 194, 234).

The classical Barbier-Wieland degradation (9, 301) of the bile acid side-

chain furnishes an overall yield of 3 to 7 per cent; attempts to improve on this have led to numerous alternative methods of degradation (63, 89, 119, 181, 182, 191). Undoubtedly the best method for degradation of the bile acid side chain (as XII) is that of Wettstein & Miescher, which eliminates three carbon atoms at one time, permits overall yields of 20 to 30 per cent, and leads as shown, to either the 20-ketone (XIII) (91, 197 to 201, 208, 299, 302) or the 21-acetoxy-20-ketone (XIV) (202, 203, 204, 299, 300).

In the case of steroids with unsaturated side chains, such as ergosterol (XV) or stigmasterol (XVII), ozonolysis leads to 22-aldehydes (XVI) convertible into enol-acetates (XVIII), which by further ozonolysis afford 20-ketones (XIII) (38, 101, 112, 113, 114). Alternatively, the 22-aldehyde (XVI) may be converted with piperidine into the enamine (XIX), which by ozonolysis affords the 20-ketone (XIII) (102).

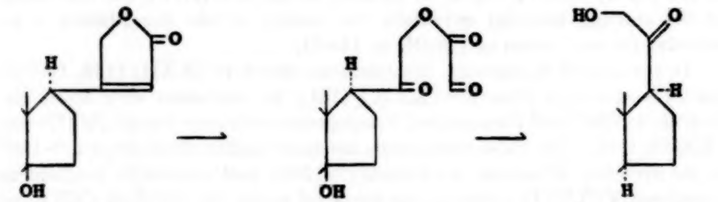
Cardiac aglycones (XX) can be degraded by ozonolysis at $-80°$ to give glyoxylic esters (XXI) which are readily hydrolysed with potassium bicarbonate to 14β:21-dihydroxy-20-ketones (194), convertible by elimination of the 14β-hydroxyl group to 21-hydroxy-20-ketones (XIV), but the rarity of the starting material outweighs the brevity of the degradation, even allowing for conversion of 14β-OH to 14α-H.

In the case of sapogenins, the spirostan structure (XXII) (159, 160) or the 22-isospirostan structure (XXIV) (161), by treatment with acetic anhydride at 200° yield the so-called Ψ-sapogenin acetates or furost-20(22)-enes (XXIII) (164, 173); these compounds are more readily obtained at 100–140° in the presence of various Lewis acids (95, 261), and are readily oxidised to afford esters (XXVI) which can be degraded to the 16β,20β-diols (XXV) or to Δ^{16}-20-ketones (XXVII) (160, 162, 163, 165, 166). It should be noted that a product described originally as allopregnane-3,12,20-trione (175) and as 17α-hydroxyallopregnane-3,12,20-trione (176, 293, 294), and prepared from Ψ-hecogenin by the process (XXIII$\rightarrow$XXVI$\rightarrow$XXVII), involving hydrolysis with methanolic potassium hydroxide, has been shown to be 16-methoxyallopregnane-3,12,20-trione (84).

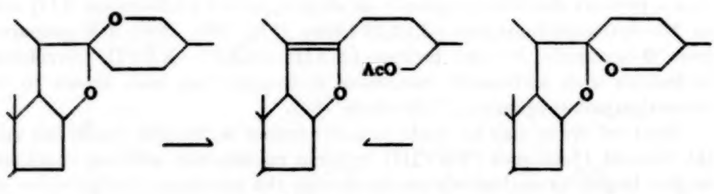
Brief reference may be made to construction of the side chains (*a*) and (*b*). Steroid 17-ketones (XXVIII) undergo nucleophilic addition reactions to give largely or exclusively products with the unnatural configuration at $C_{(17)}$ (233, 251); the only exception appears to be cyanhydrin formation, but the difficulty may be evaded in various ways. The Darzens reaction (46, 47) with dehydroepiandrosterone (as XXVIII) and ethyl $\alpha\alpha$-dichloropropionate leads to a 17-*iso*-ester (XXIX), which after saponification undergoes decarboxylation and rearrangement (XXX) with inversion of configuration to give the 17-*n*-20-ketone (XIII) accompanied by the D-homo-isomeride (207, 310). The epimeric cyanhydrins derived from a 17-ketone (XXVIII) (31, 108, 139, 140, 193, 205) by dehydration to the Δ^{16}-nitrile (XXXI), hydrolysis of this to the Δ^{16}-acid (XXXII), and hydrogenation furnish the 17-*n*-acid (XXXIII) (31, 226, 257); this may be converted as the acid chloride by cadmium dimethyl into the 20-ketone (XIII) (195, 283), and by the

diazoketone synthesis into either the 20-ketone (XIII) or the 21-acetoxy-20-ketone (XIV) (226, 305). The diazoketone synthesis cannot be applied to the Δ^{16}-acid (XXXII) (256), but the action of methylmagnesium bromide on the Δ^{16}-nitrile (XXXI) gives the Δ^{16}-ketone (XXVII) (33). Addition of organometallic compounds, e.g., potassium acetylide, to 17-ketones (XXVIII) gives 17α-ethynyl derivatives (252, 282) hydrogenated by palladium to 17α-vinyl compounds (XXXIV) (253); one such with osmium tetroxide furnishes the 17β,20β,21-triol (XXXV) which, as the diacetate, undergoes the Serini reaction to give, with inversion of configuration, the 21-acetoxy-20-ketone (XIV) (34, 75, 235, 277, 278, 280).

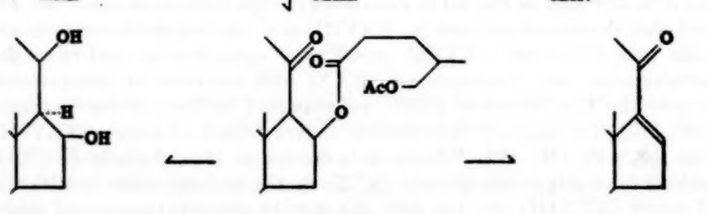
The only efficient routes to 17α-hydroxy steroids are from *cis-* or *trans-* Δ^{17}-steroids or from Δ^{16}-steroids. Δ^{17}-steroids (XLI) are available by dehydration of 17-*iso*-17β-hydroxysteroids (34), by anionotropic change of 17α-vinyl-17β-hydroxy compounds (XXXIV) (151, 254, 255, 262, 275, 277), by Wolff-Kishner reduction of semicarbazones of Δ^{16}-20-ketones (XXVII) (80), or as the enol-acetates of 20-ketones (XIII) (12, 50, 56, 106, 137, 177, 210,



XX XXI XIV

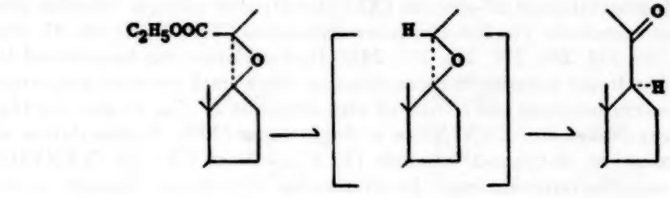


XXII XXIII XXIV

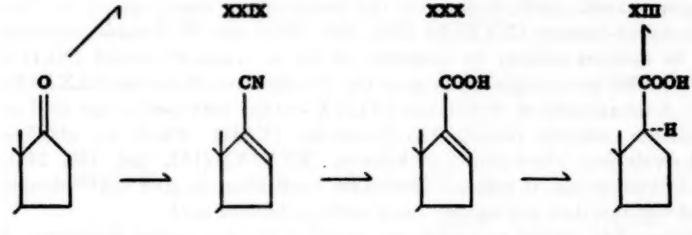


XXV XXVI XXVII

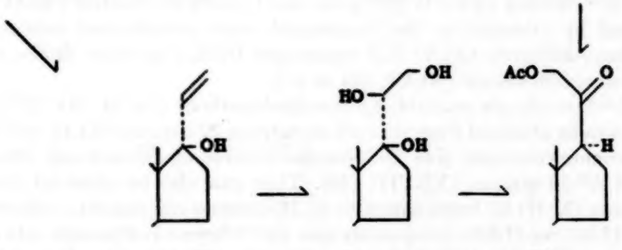
217, 291). They can also be obtained by the Favorsky reaction (150); a 20-ketone (XIII) is converted into the 17,21,21-tribromo derivative (XXXVI), rearranged by alkali to the bromo acid (XXXVII), which is reduced as the



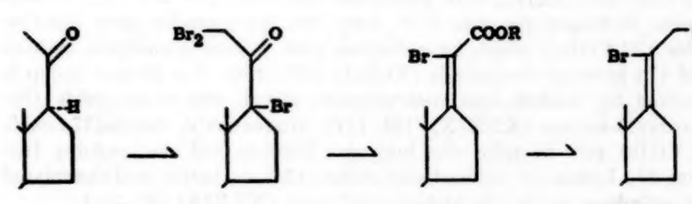
XXIX XXX XIII



XXVIII XXXI XXXII XXXIII



XXXIV XXXV XIV



XIII XXXVI XXXVII XXXVIII

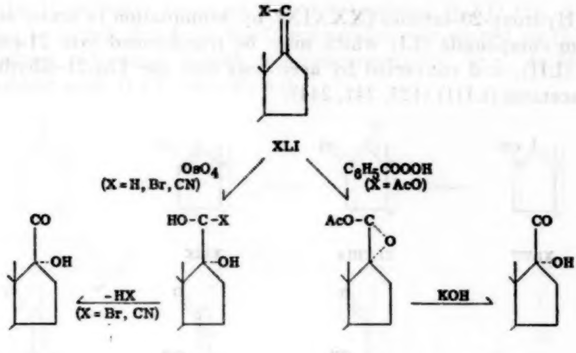
methyl ester with lithium aluminium hydride to the Δ^{17}-21-hydroxy-20-bromo compound (XXXVIII) (296). Δ^{17}-steroids of rather special types have also been described (81, 82, 261).

Hydroxylation of Δ^{17}-steroids (XLI:X=H) with osmium tetroxide give almost exclusively 17α,20β-dihydroxy derivatives (XL:X=H) (66, 97, 206, 227, 228, 233, 258, 275, 276, 277, 287). Hydroxylation can be achieved by use of hydrogen peroxide in the presence of only a small catalytic proportion of osmium tetroxide and combined with oxidation of $C_{(20)}$ to give the 17α-hydroxy-20-ketone (XXXIX) in a single stage (209). Hydroxylation of 20-bromo- or 20-cyano-Δ^{17}-steroids (XLI:X=Br or CN) (cf. XXXVIII) with osmium tetroxide leads, by elimination of hydrogen bromide or hydrogen cyanide on hydrolysis of the intermediate osmic esters, to 17α-hydroxy-20-ketones (XXXIX) (263, 295, 297); use of osmium tetroxide can be avoided entirely by oxidation of the 20-cyano-Δ^{17}-steroid (XLI:X=CN) with permanganate to give the 17α-hydroxy-20-ketone (XXXIX) (96). Enol-acetates of 20-ketones (XLI:X=OAc) with perbenzoic acid or chromium trioxide furnish 17α,20α-oxides (XLII), which by alkaline hydrolysis give 17α-hydroxy-20-ketones (XXXIX) (132, 134, 136, 244). An 11-keto group, if present, undergoes enolisation to give a $\Delta^{9:11}$-double bond but this does not readily react with perbenzoic acid.

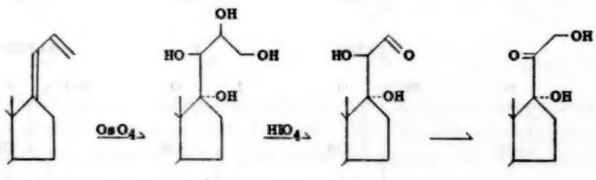
Somewhat special examples are provided by the partial syntheses of Reichstein's substances P and S; hydroxylation with osmium tetroxide of the $\Delta^{17,21}$-steroid (XLIII) (32) gives the 17α,20β,21,22-tetrol (XLIV), converted by oxidation as the 20-acetonide with periodic acid into the 20β-hydroxy-aldehyde (XLV) and rearranged by hot pyridine to the 17α,21-dihydroxy-20-ketone (XLVI) (64 to 67).

Δ^{16}-Steroids are available by standard methods (33, 93, 219, 279). They can also be obtained from the enol acetates of 20-ketones (XLI), which with N-bromosuccinimide give 16-bromides capable of anionotropic change to yield Δ^{16}-20-ketones (XXVII) (49). They can also be obtained from 20-ketones (XIII) by bromination to 17,21-dibromo compounds, converted via the 17-bromo-21-iodo compounds and the 17-bromo compounds into Δ^{16}-20-ketones (XXVII) (126, 131, 167, 168, 169, 171, 172, 174, 222). The best source of Δ^{16}-steroids appears undoubtedly to be the furost-20(22)-enes (XXIII), derived from the steroid sapogenins.

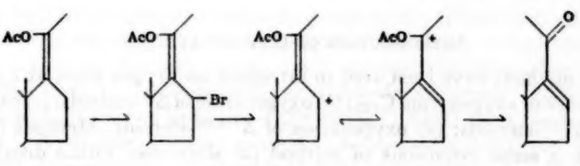
Δ^{16}-Steroids (XLIX), with perbenzoic acid (127, 170, 221, 224) or with alkaline hydrogen peroxide (128, 130, 239, 241), readily give 16α,17α-oxides (XLVIIIa), which by reduction with lithium aluminium hydride yield 17α-hydroxy compounds (XLVII) (127, 224); if a 20-keto group is protected by condensation with ethylene glycol, this route yields 17α-hydroxy-20-ketones (XXXIX) (124, 127). Alternatively, the 16α,17α-oxide (XLVIIIb) may be split with hydrogen bromide and the resulting 16β-bromo-17α-hydrin (L) reduced with nickel (125), or, better, hydrogenolysed with palladium to the 17α-hydroxy-20-ketone (XXXIX) (43, 241).



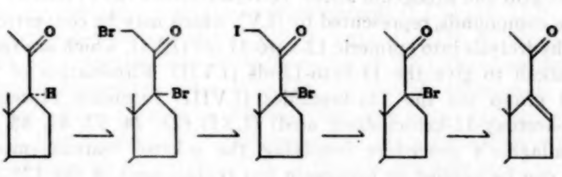
XXXIX XL XLII XXXIX



XLIII XLIV XLV XLVI

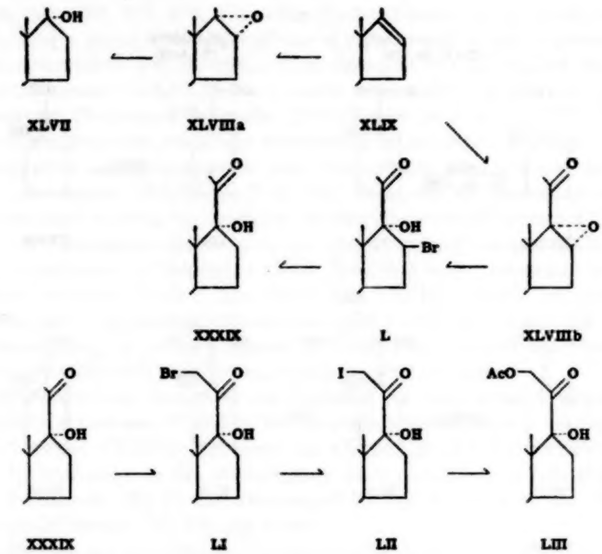


XLI XXVII



XIII XXVII

17α-Hydroxy-20-ketones (XXXIX), by bromination in acetic acid, give 21-bromo compounds (LI) which may be transformed into 21-iodo compounds (LII), and converted by acetolysis into the 17α,21-dihydroxy-20-ketone acetates (LIII) (125, 241, 244).



XLVII XLVIIIa XLIX

XXXIX L XLVIIIb

XXXIX LI LII LIII

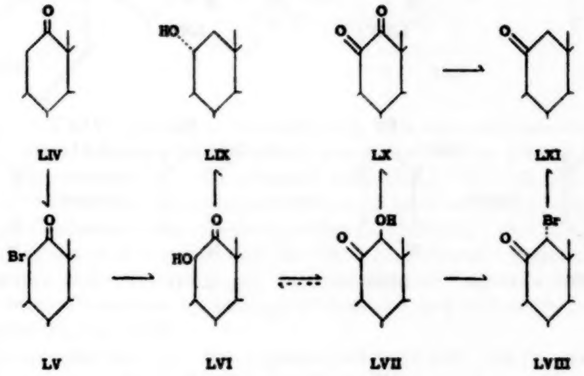
Introduction of Oxygen at $C_{(11)}$

Four methods have been used to introduce an oxygen atom at $C_{(11)}$: (*a*) transference of oxygen from $C_{(12)}$; (*b*) oxygenation of Δ^{11}-steroids; (*c*) oxygenation of $\Delta^{9(11)}$-steroids; (*d*) oxygenation of $\Delta^{7:9(11)}$-steroids. Methods (*b*) and (*c*) are in a sense extensions of method (*a*) since they utilize deoxycholic acid as starting material.

(*a*) Steroids oxygenated at $C_{(12)}$ are readily available as natural products in the bile acid and sapogenin series. 12-Ketosteroids (LIV) furnish epimeric 11-bromo compounds, represented by (LV), which may be converted by mild alkaline hydrolysis into epimeric 12-keto-11-ols (LVI), which are rearranged by hot alkali to give the 11-keto-12-ols (LVII). Elimination of the 12β-hydroxyl group via the 12α-bromides (LVIII) furnishes 11-ketosteroids (e.g., 3α-acetoxy-11-ketocholanic acid) (LXI) (23, 24, 52, 87, 88, 90, 104, 152). Gallagher's procedure involving the α-ketol rearrangement (LVI ⇌ LVII) can be applied to hecogenin but replacement of the 12β-hydroxyl group leads to attack in the spiroketal side chain, and elimination is achieved by oxidation to the 11,12-diketone (LX) followed by Wolff-Kishner reduc-

tion of the 12-carbonyl group to the 11-ketosteroid (LXI) (51, 52, 211).

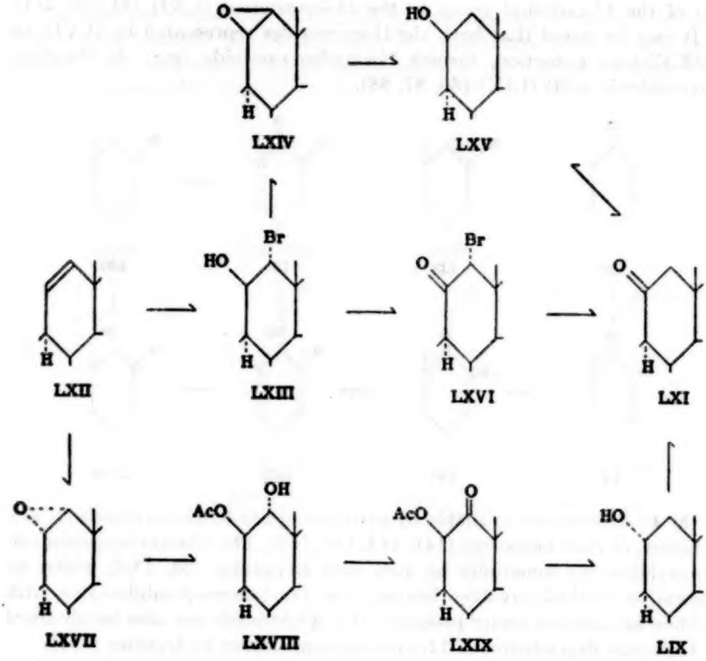
It may be noted that both the 11-epimerides represented by (LVI), by Wolff-Kishner reduction, furnish 11α-hydroxysteroids (e.g., 3α,11α-dihydroxycholanic acid) (LIX) (86, 87, 88).



(*b*) k^{11}-Steroids are available by pyrolysis of 12α-hydroxysteroids (1, 27), or, better, of their benzoates (141, 143, 145, 189). The 12α-anthraquinone-β-carboxylates can sometimes be used with advantage (68, 146), whilst an alternative method involves heating the 12α-toluene-*p*-sulphonates with pyridine or collidine under pressure (70). Δ^{11}-Steroids can also be obtained by Hofmann degradation of 12-trimethylammonium hydroxides (191).

Δ^{11}-Steroids (LXII), by Reichstein's procedure, furnish 12α-bromo-11β-hydrins (LXIII), a process catalysed by traces of sulphuric acid (262); the bromohydrins are difficult to isolate, but are converted by treatment with alkali or filtration through aluminium oxide into 11β,12β-oxides (LXIV); the latter may be hydrogenated to 11β-hydroxysteroids (LXV) (215). Oxidation of the crude 12α-bromo-11β-hydrins (LXIII) gives 12α-bromo-11-ketones (LXVI) debrominated to yield 11-ketosteroids (LXI) (68, 142, 143, 145, 225). Alternatively, Δ^{11}-steroids (LXII) with perbenzoic acid give 11α, 12α-oxides (LXVII) which undergo acetolysis to 11β-acetoxy-12α-hydroxysteroids (LXVIII), oxidised to 11β-acetoxy-12-ketones (LXIX), which by Wolff-Kishner reduction give 11α-hydroxysteroids (LIX) (86, 152).

3α-Hydroxy-Δ^{11}-steroids of the A/B-*cis* series (LXII), by the procedure of Kendall, are converted into Δ^{11}-3α,9α-oxides (LXX); these normally yield two isomeric *trans*-dibromides [11α,12β, equatorial, equatorial; 11β,12α, polar, polar (LXXI).] Owing to the polar character of the 11β-bromine atom in (LXXI), this is labile and undergoes substitution by anions with retention of configuration by participation of the 12α-bromine atom to give the 12α-bromo-11β-hydrin (LXXII). Oxidation to the bromoketone (LXXIII) and removal of the 12α-bromine atom by reduction furnishes the 11-keto-3α,


LXIV
LXV
LXII
LXIII
LXVI
LXI
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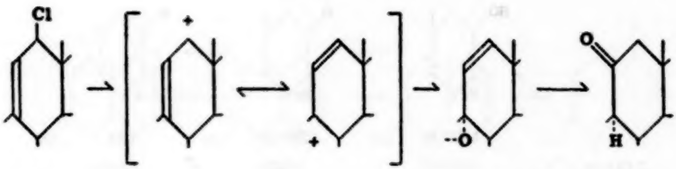
LXI
LVIII
LXXIV
LXXIII



LXXV LXX LXI

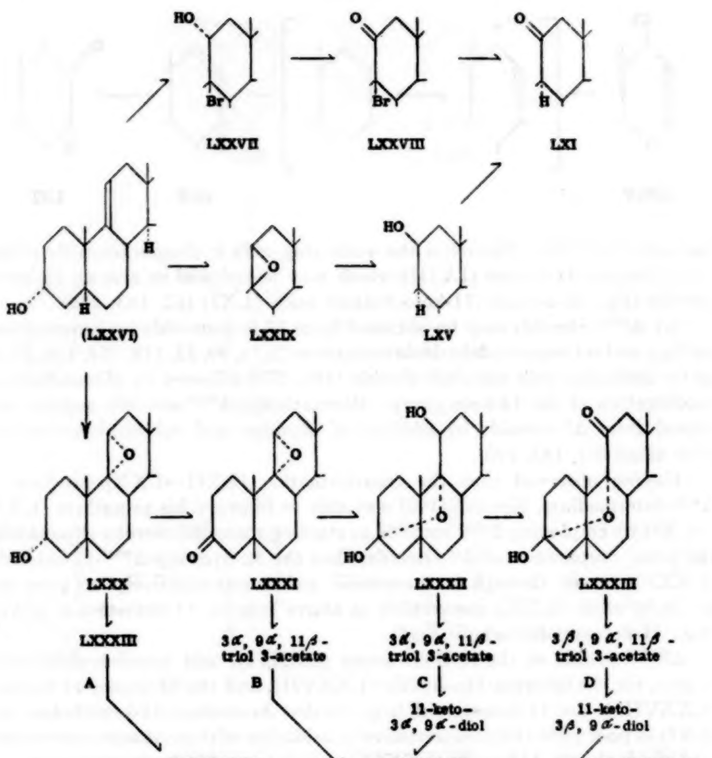
9α-oxide (LXXIV). Fission of the oxide ring with hydrogen bromide yields the 12-bromo-11-ketone (LVIII) which may be reduced to give an 11-ketosteroid (e.g., 3α-acetoxy-11-ketocholanic acid) (LXI) (62, 183, 290).

(*c*) $\Delta^{9(11)}$-Steroids may be obtained from 12-ketosteroids by bromination at $C_{(11)}$ and subsequent dehydrobromination (2, 15, 39, 52, 118, 153, 158, 271), or by oxidation with selenium dioxide (190, 270) followed by elimination or modification of the 12-keto group. Alternatively, $\Delta^{9(11)}$-steroids may be obtained from Δ^{11}-steroids by addition of bromine and dehydrobromination with alkali (62, 183, 290).

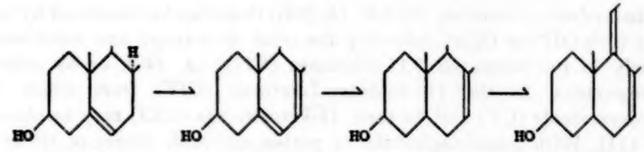
Having observed that the transformation (LXII→LXX) involves a $\Delta^{9(11)}$-intermediate, Kendall (190) was able to improve his procedure (LXX→LXI) by employing $\Delta^{9(11)}$-steroids as starting material, thereby eliminating the prior preparation of Δ^{11}-steroids; thus the 3α-hydroxy-$\Delta^{9(11)}$-12-chloride (LXXV) passes through a mesomeric anion quantitatively to give the Δ^{11}-$3\alpha,9\alpha$-oxide (LXX), convertible as above into an 11-ketosteroid (LXI) (e.g., 11-ketoaetiolithocholic acid).

$\Delta^{9(11)}$-Steroids of the A/B-*cis* series (LXXVI), add hypobromous acid to give, via the 9β-brom-11α-hydrin (LXXVII), and the 9β-bromo-11-ketone (LXXVIII), the 11-ketosteroid (e.g., methyl 3α-acetoxy-11-ketocholanate) (LXI) in poor yield (118). Alternatively, oxidation with permanganate-acetic acid yields the 9β, 11β-oxide (LXXIX), which is readily hydrogenated to an 11β-hydroxysteroid (e.g., methyl-3α-acetoxy-11β-hydroxycholanate) (LXV), and oxidised to an 11-ketosteroid (LXI) (44, 117); this reaction sequence fails for A/B-*trans* $\Delta^{9(11)}$-steroids (44). $\Delta^{9(11)}$-Steroids (LXXVI) with peracids afford 9α, 11α-oxides (as LXXX) (2, 15, 77, 177, 271); if these are of the A/B-*cis* series and possess a 3α-hydroxy group (LXXX), oxidation with chromic acid leads to the corresponding 3-ketone (LXXXI), which shows remarkable reactivity in solvolytic reactions and is hydrolysed by mineral acid to give the 11β-hydroxy-$3\alpha,9\alpha$-hemiketal (LXXXII); the latter, by oxidation, yields the 11-keto-$3\alpha,9\alpha$-hemiketal (LXXXIII) (76, 115). Four routes (A, B, C, D) are available for conversion of such hemiketals into 11-ketosteroids [e.g., methyl-3,11-diketocholanate (LXIa), methyl-3α-acetoxy-11-ketocholanate (LXIb), and 3β-acetoxy-11-ketocholanate (LXIc); methyl 3,11-diketoaetiocholanate, (LXI)] (115, 116).

(*d*) $\Delta^{7:9(11)}$-Steroids (LXXXVII) are obtained by dehydrogenation of Δ^{7}-steroids (LXXXVI) with mercuric acetate (5, 10, 53, 78, 98, 107, 109, 239,



247, and 307) or with bromine (3). A Δ^7-linking is present in ergosterol (as LXXXV), and Δ^5-steroids (LXXXIV) are readily converted into $\Delta^{5:7}$-steroids (LXXXV) with bromine (266, 267, 268), or preferably, by use of N-bromosuccinimide (4, 16, 17, 18, 20, 53, 246, 285, 286). Reduction of $\Delta^{5,7}$-steroids (LXXXV) to Δ^7-steroids (LXXXVI) can be accomplished quantitatively (3, 53, 109, 148, 212, 247). Numerous papers dealing with the preparation



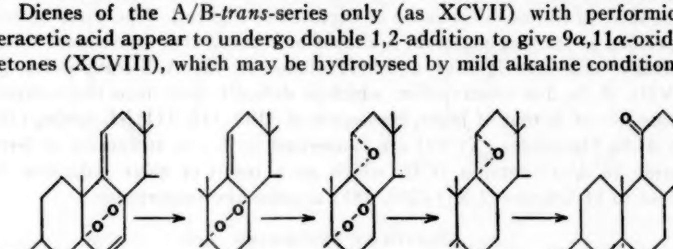
LXXXIV LXXXV LXXXVI LXXXVII

of $\Delta^{7:9(11)}$-steroids have appeared recently in addition to those cited above (7, 107, 212, 237, 239, 311).

W. Bergmann in 1948 considered the conversion of ergosterol (as LXXXV) via dehydroergosterol (as LXXXVII) into an 11-oxygenated steroid (13, 14) but protection of the conjugated nuclear double bond system by addition of oxygen or maleic anhydride was necessary, and the resulting peroxide or maleic anhydride structure could not readily be eliminated at a later stage (6, 8, 149). Jones *et al.* have recently used the peroxide of dehydroergosterol (LXXXVIII) by preferential reduction of the Δ^6-double bond (LXXXIX), $9\alpha,11\alpha$-oxide formation (XC) followed by hydrogenolysis to the $5\alpha,8\alpha$-diol (XCI) which furnishes 5α-hydroxy-11-ketosteroids (XCII) (21, 22, 99).

Oxidation of $\Delta^{7,9(11)}$-steroids (XCVII) may conveniently be regarded as involving 1,4-[6] or 1,2-addition to the diene system. Oxidation with dichromate of dienes of either the A/B-*cis*- or the A/B-*trans*-series by a 1,4-process yields Δ^8-7,11-diketones (CII), reduced readily to 7,11-diketones (CIII), from which the 7-carbonyl group is removed by Wolff-Kishner reduction or by the thioketal-nickel procedure (78). Alternatively, use of N-bromoacetamide leads, by 1,4-addition of hypobromous acid, to the Δ^8-11α-brom-7β-hydrin, which is oxidised by excess of the reagent to the Δ^8-11α-bromo-7-ketone (XCIV); the latter may be hydrolysed to the Δ^8-11α-hydroxy-7-ketone (XCIX), which can be oxidised to the Δ^8-7,11-diketone (CII) (79).

Dienes of the A/B-*trans*-series only (as XCVII) with performic or peracetic acid appear to undergo double 1,2-addition to give $9\alpha,11\alpha$-oxido-7-ketones (XCVIII), which may be hydrolysed by mild alkaline conditions to



LXXXVIII LXXXIX XC XCI XCII

[6] The process 7,9(11)-diene→7,11-diketo-8-ene→7,11-diketone may be compared with the conversion of cholesta-3,5-diene to cholestane-3,6-dione carried out by Windaus in 1906 (306).

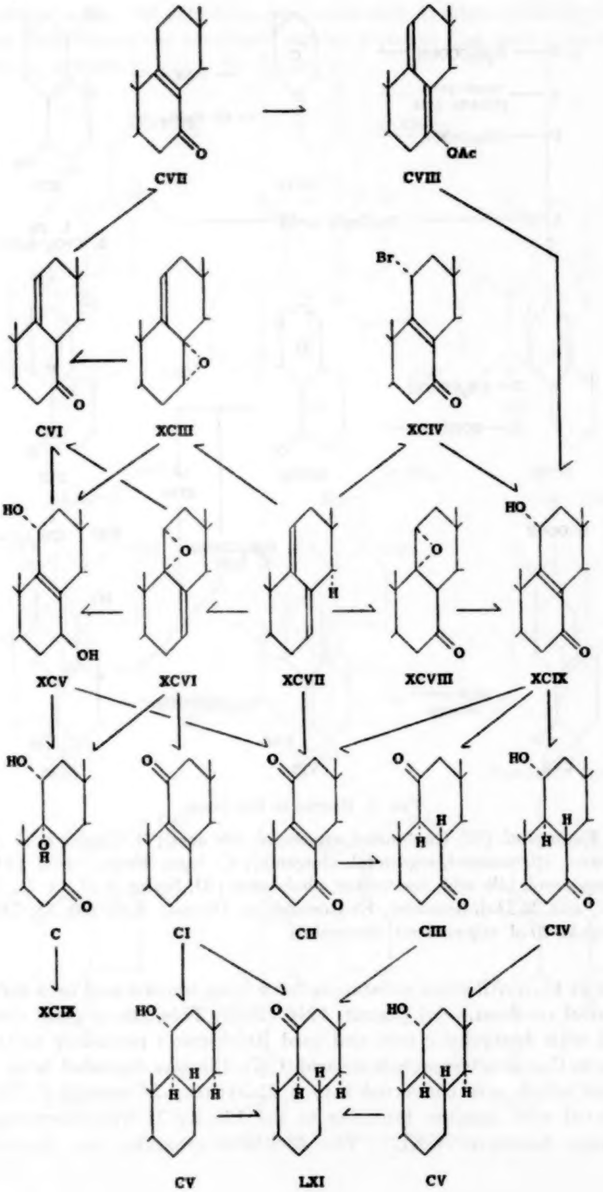
Δ^8-11α-hydroxy-7-ketones (XCIX) (3, 284); these can be converted by treatment with OH^- or OBu^t, following the triad prototropy and ketonisation, directly to the saturated 7,11-diketones (CIII) (3, 240), or by selective hydrogenation to the 11α-hydroxy-7-ketones (CIV) from which 11α-hydroxysteroids (CV) and, in turn, 11-ketosteroids (LXI) may be obtained (55, 111). With permonophthalic or perbenzoic acid, dienes of either the A/B-*cis*-or the A/B-*trans*-series give mainly the Δ^7-9α,11α-oxides (XCVI), convertible by mild alkaline hydrolysis to Δ^8-7β,11α-diols (XCV), which furnish by further oxidation the Δ^8-7,11-diketones (CII) (40, 78, 109, 110, 111), or, by further peroxidation, hydrolysis, and dehydration, the 9α,11α-dihydroxy-7-ketones (C) (25, 111) further dehydrated to Δ^8-11α-hydroxy-7-ketones (XCIX). Alternatively the Δ^7-9α,11α-oxides (XCVI), by treatment with boron trifluoride or ferric chloride (109), furnish Δ^8-11-ketones (CI), from which, by reduction with lithium, 11-ketosteroids (LXI) are obtained; the use of lithium-ethanol, however, leads to 11α-hydroxysteroids (CV) (109, 269, 281). By brief treatment with acid, the Δ^7-9α,11α-oxides (XCVI) and the Δ^8-7β,11α-diols (XCV) give $\Delta^{9(11)}$-7-ketones (CVI) (109, 110, 111, 269, 281), which are byproducts in the performic acid oxidation of dienes of the A/B-*trans* series and of dichromate oxidation of dienes of both the A/B-*trans*-and the A/B-*cis*-series.

Performic acid oxidation of dienes of the A/B-*trans*-series (cf. above) or dichromate oxidation of dienes of both the A/B-*trans*-and the A/B-*cis*-series furnishes as byproducts, apparently by 1,2-addition, $\Delta^{9(11)}$-7α,8α-oxides (XCIII) and $\Delta^{9(11)}$-7-ketones (CVI); these are readily rearranged to Δ^8-7-ketones (CVII), which form the exclusive product of performic acid oxidation of dienes of the A/B-*cis*-series (3, 50, 78, 79, 111). Such Δ^8-7-ketones (CVII) of either the A/B-*trans*- or the A/B-*cis*-series give enol-acetates (CVIII) which by oxidation with perbenzoic acid yield directly the Δ^8-11α-hydroxy-7-ketones (XCIX) (50, 55).

The more significant reactions, of those just discussed, can be extracted in the form of routes to cortisone as depicted in Figure 2. Where a Δ^6-steroid is specified as starting material, it should be realised that subsequent transformation converts it into a 7,9(11)-diene of the A/B-*trans*-series (as XCVII). A further observation, which is difficult to fit into the compact scheme shown, is that of Jeger, Heusser *et al.* (109, 110, 111) [cf. Spring (26)] that Δ^7-9α,11α-oxides (XCVI) are isomerised by boron trifluoride or ferric chloride to Δ^8-11-ketones (CI), which as a result of their reduction by lithium to 11-ketones (LXI) (269, 281) acquire new importance.

Cortisone Syntheses

The foregoing sections suggest that the principal difficulty in the synthesis of cortisone remains that of introduction of oxygen at $C_{(11)}$, but that this can be overcome in a variety of ways. The most suitable natural products, available in quantity, for cortisone syntheses appear to be: (*a*) bile acids and hecogenin for routes involving transfer of oxygen from $C_{(12)}$ to $C_{(11)}$, (*b*) ergosterol, stigmasterol, and diosgenin for routes requiring introduction of


CVII
CVIII
OAc
Br
CVI
XCIII
XCIV
HO
OH
XCV
XCVI
XCVII
XCVIII
XCIX
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XCIX
CV
LXI
CV

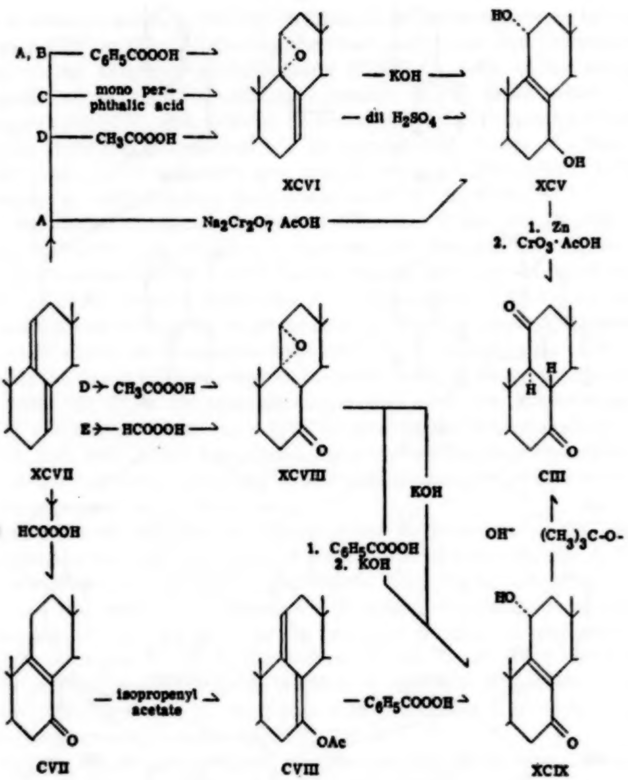


FIG. 2. Routes to Cortisone.

[A: Fieser *et al.* (78) (cholesterol, ergosterol, bile acid); B: Tishler *et al.* (40, 41) (cholesterol, stigmasterol, ergosterol, diosgenin); C: Jeger, Heusser *et al.* (109, 110, 111) (ergosterol, bile acid, androstane, cholestane); D: Spring *et al.* (3, 25, 26) (ergosterol, and 22,23-di-bromide); E: Rosenkranz, Djerassi *et al.* (50, 55, 240, 284) (allopregnan-20-ol, stigmasterol, diosgenin)]

oxygen at $C_{(11)}$. All these substances have been transformed into cortisone.

Partial syntheses.—(*a*) [Sarett, 1946 (262)]. This, the original synthesis started with deoxycholic acid and used Reichstein's procedure to transfer oxygen to $C_{(11)}$ in a bisnorcholanic acid (CX); this was degraded to an 11,17-diketone which was converted into a 21-hydroxy-Δ^{17}-steroid (CXI), hydroxylated with osmium tetroxide to the 17α,20β,21-trihydroxypregnane-3,11-dione diacetate (CXII). The Δ^4-4-keto grouping was inserted by

bromination at $C_{(4)}$ and dehydrobromination with pyridine (CXIII); finally, cautious oxidation of the secondary alcohol group at $C_{(20)}$ gave a poor yield of cortisone acetate (CXIV). See Figure 3.

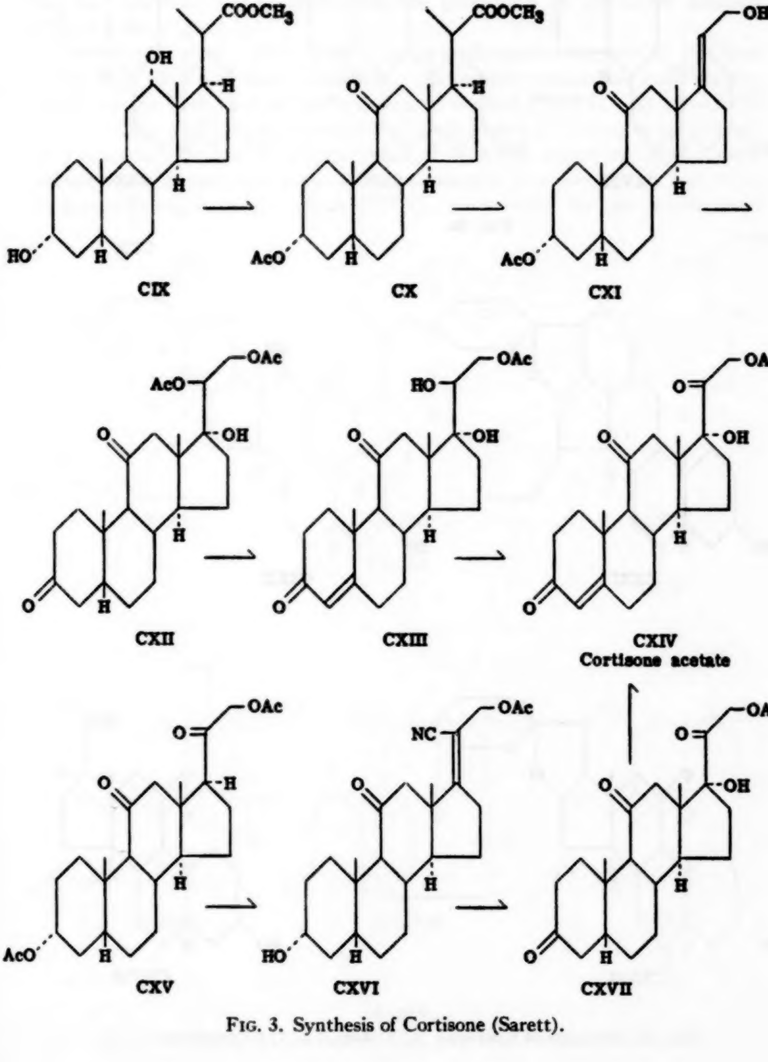
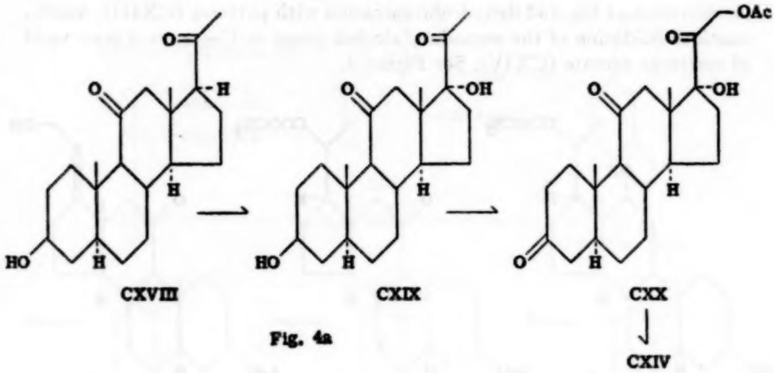
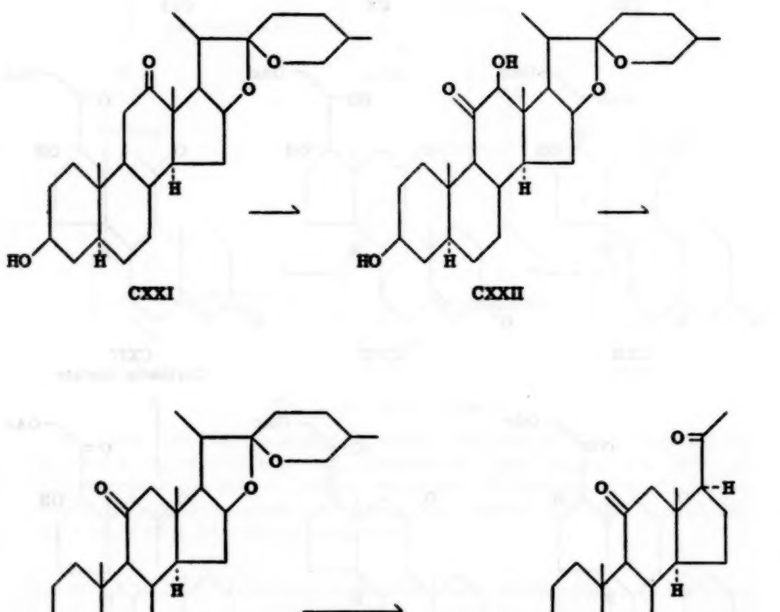


FIG. 3. Synthesis of Cortisone (Sarett).



CXVIII CXIX CXX → CXIV

Fig. 4a



CXXI CXXII

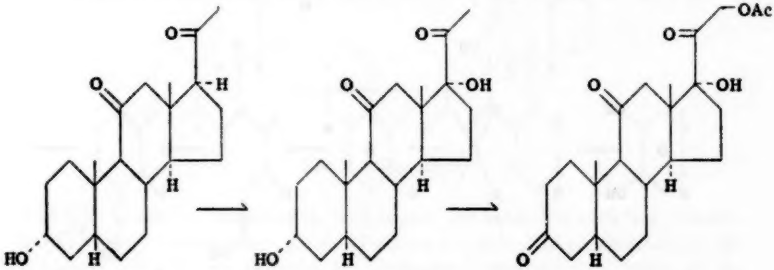
CXXIII CXVIII

FIG. 4b

FIG. 4. Synthesis of Cortisone (a: Tishler *et al.*; b: Rosenkranz *et al.*)

(*b*) [Sarett, 1948 (263)]. The 11-ketosteroid (CXV), derived from deoxycholic acid by Kendall's improved procedure, was converted into the 20-cyano-Δ^{17}-steroid (CXVI), hydroxylated with osmium tetroxide to furnish the dihydroxy acetone side chain in the triketone (CXVII), and converted by the Mattox-Kendall arylhydrazone procedure to cortisone acetate (CXIV). See Figure 3.

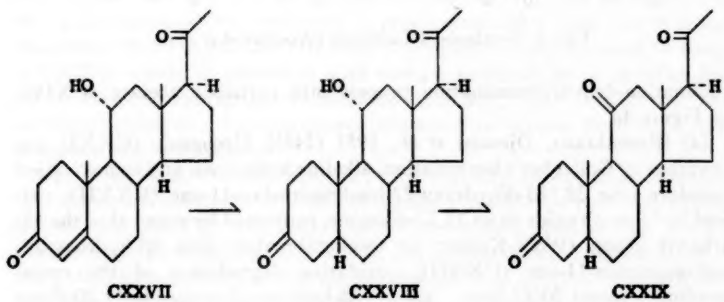
(*c*) [Tishler *et al.*, 1951 (41)]. The 3β-hydroxyallopregnane-11,20-dione (CXVIII), obtained from diosgenin, cholesterol, ergosterol, and stigmasterol, by the 7,9(11)-diene-perbenzoic acid method (XCVII→XCVI→XCV →CII→CIII→LXI) was converted by Gallagher's enol acetate-perbenzoic acid procedure into the 17α-hydroxyketone (CXIX), which by bromination, acetolysis, and oxidation with N-bromacetamide furnished 21-acetoxy-17α-hydroxyallopregnane-3,20-trione (CXX), convertible by an undisclosed



CXXIV CXXV CXXVI

CXIV

Fig. 5a



CXXVII CXXVIII CXXIX

CXIV

Fig. 5b

Fig. 5. Synthesis of Cortisone (a: Gallagher *et al.*; b: Rosenkranz *et al.*).

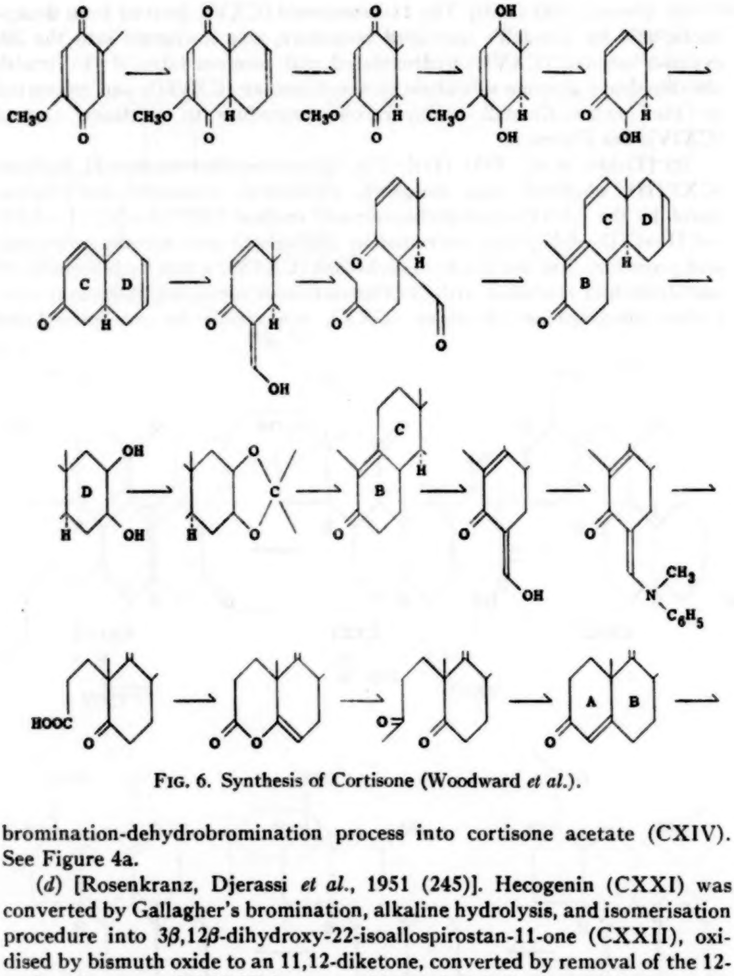


FIG. 6. Synthesis of Cortisone (Woodward *et al.*).

bromination-dehydrobromination process into cortisone acetate (CXIV). See Figure 4a.

(*d*) [Rosenkranz, Djerassi *et al.*, 1951 (245)]. Hecogenin (CXXI) was converted by Gallagher's bromination, alkaline hydrolysis, and isomerisation procedure into 3β,12β-dihydroxy-22-isoallospirostan-11-one (CXXII), oxidised by bismuth oxide to an 11,12-diketone, converted by removal of the 12-carbonyl group (Wolff-Kishner or thioketal-nickel) into 3β-hydroxy-22-isoallospirostan-11-one (CXXIII); oxidative degradation of the corresponding furost-20(22-)ene gave 3β-hydroxyallopregnane-11,20-dione (CXVIII) [See Figure 4b and partial synthesis (*c*)].

(*e*) [Gallagher *et al.*, 1952 (137)]. 3β-Hydroxypregnane-11,20-dione (CXXIV), from deoxycholic acid, was converted by Gallagher's method, using the enol acetate and perbenzoic acid, into the 17α-hydroxyketone

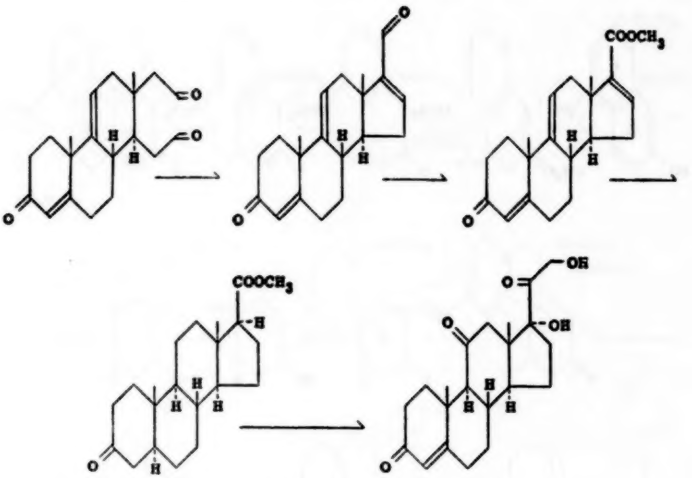


FIG. 6 (continued)

(CXXV) which by bromination, hydrolysis, and oxidation with N-bromacetamide, and acetylation, gave 21-acetoxy-17α-hydroxypregnane-3,11,20-trione (CXXVI), transformed by Gallagher's modification (133) of the Mattox-Kendall arylhydrazone method into cortisone acetate (CXIV). See Figure 5a.

(*f*) [Rosenkranz, Djerassi *et al.*, 1952 (156)]. 11α-Hydroxyprogesterone (CXXVII), obtained in 45 per cent yield by biochemical oxygenation of progesterone (83, 156, 218) or by partial synthesis (157), on hydrogenation with palladium-charcoal preferably in presence of OH^-, gives 11α-hydroxypregnane-3,20-dione (CXXVIII); this is oxidised to pregnane-3,11,20-trione (CXXIX), which by reduction with sodium borohydride in pyridine furnishes 3α-hydroxypregnane-11,20-dione (CXXIV) [see Figure 5a and partial synthesis (*e*)]. In consequence 10 steps from progesterone or 14 from diosgenin lead to cortisone.

(*g*) Fried *et al.* and Haines *et al.*, 1952 (42, 83). Biochemical oxidation of Reichstein's substance S, which is readily available by partial synthesis (65, 67, 96, 125, 132, 209, 262), gives a 25 per cent yield of 11α, 17α,21-trihydroxypregn-4-ene-3,20-dione (83); this substance, similarly obtained in small yield from substance S by Haines *et al.* (42), on acetylation and oxidation with chromium trioxide affords a 70 per cent yield of cortisone acetate.

It is extremely difficult to distinguish between the capacities for large scale development of partial syntheses (*c*) to (*g*); it is common knowledge that Sarett's partial synthesis of 1948 has been subjected to intensive development by the Merck organisation with considerable success, and cortisone

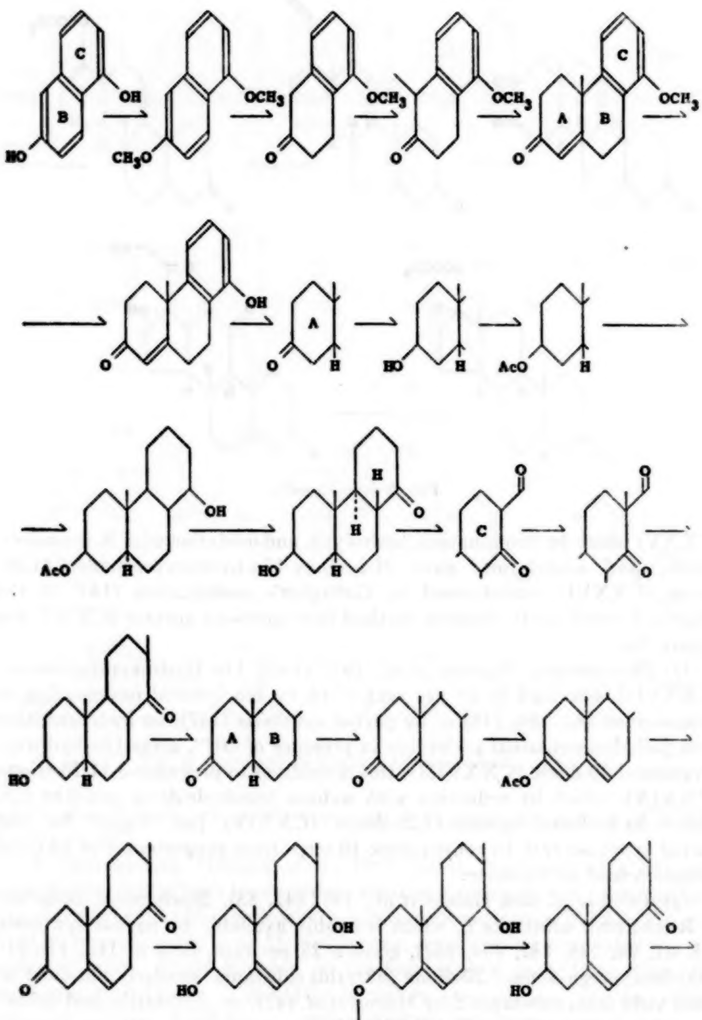


FIG. 7. Synthesis of Cortisone (Robinson *et al.*).

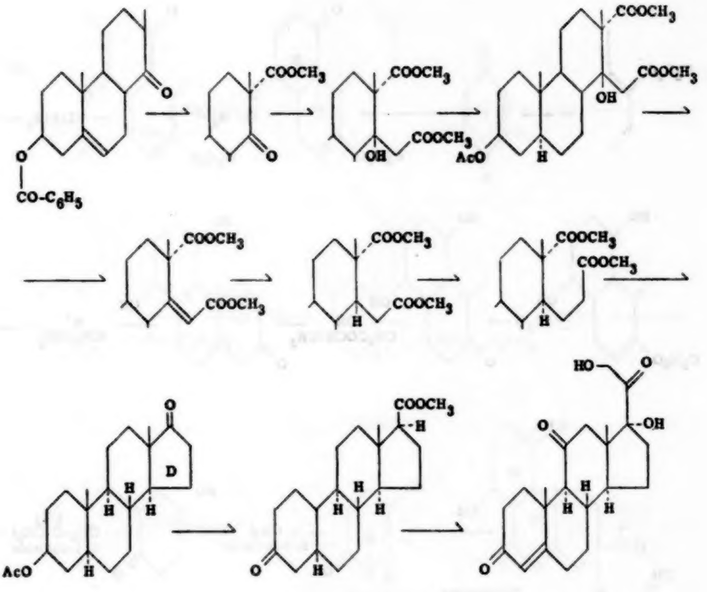


FIG. 7 (continued)

available commercially today is made by this process which starts from bile acids. The partial syntheses involving biochemical oxygenation are attractive by reason of their relative brevity; since the starting materials are now rather readily available, they may in time acquire great industrial significance, especially if yields can be improved by use of other strains of microorganisms.

Finally it may be mentioned that the synthesis of radiocortisone containing tritium, mainly at C_{16}, has been described by Gallagher (85).

Total Syntheses.—(*a*) [Woodward, *et al.*, 1952 (309)]. The DC→B→A total synthesis of methyl 3-ketoaetiochola-4,9(11),16-trienate, which has been outlined by Samuels & Reich (259), leads to that of cortisone; the detailed stages are set out as Figure 6.

(*b*) [Robinson *et al.*, 1952 (37, 45)]. As pointed out by Rosenkranz, Pataki & Djerassi (245), this follows from the A←BC→D total synthesis of epiandrosterone; this has been outlined by Samuels & Reich (259). The detailed stages are set out as Figure 7.

(*c*) [Sarett, 1952 (19, 154, 264, 265)]. The details of this short A←BC→D stereospecific total synthesis, the early stages of which recall an earlier approach by Robinson (192), are given in Figure 8, having most kindly been supplied privately by Dr. L. H. Sarett.

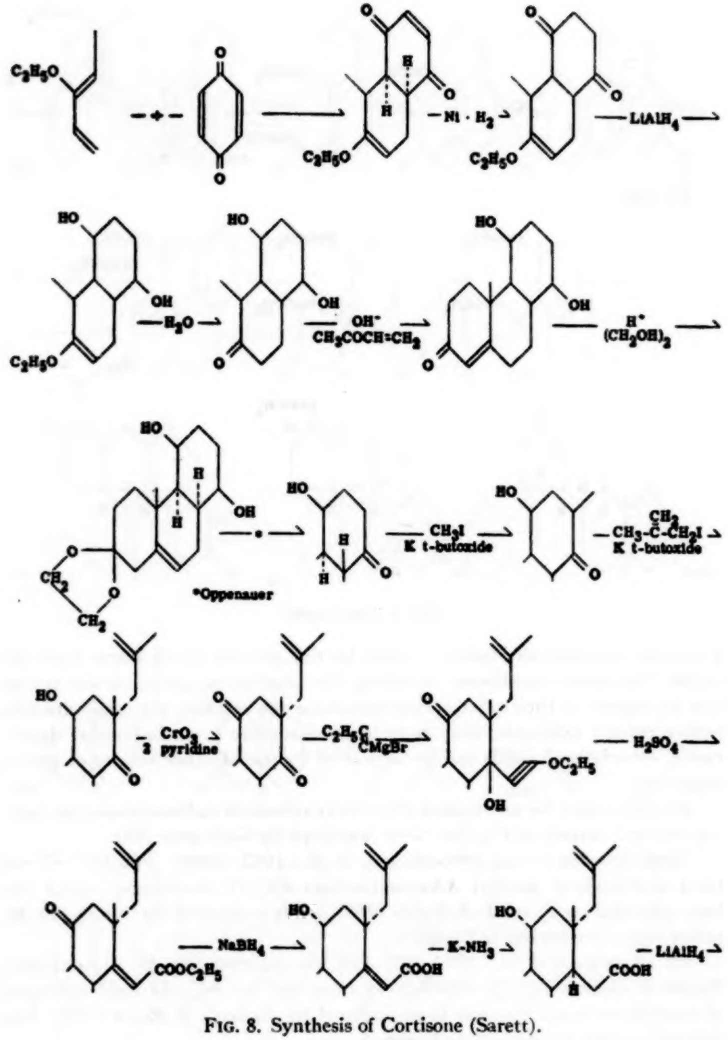


FIG. 8. Synthesis of Cortisone (Sarett).

CORTISOL

Cortisol, a name which seems convenient and preferable to hydrocortisone (by elimination of confusion with 4,5-dihydrocortisone), is the only

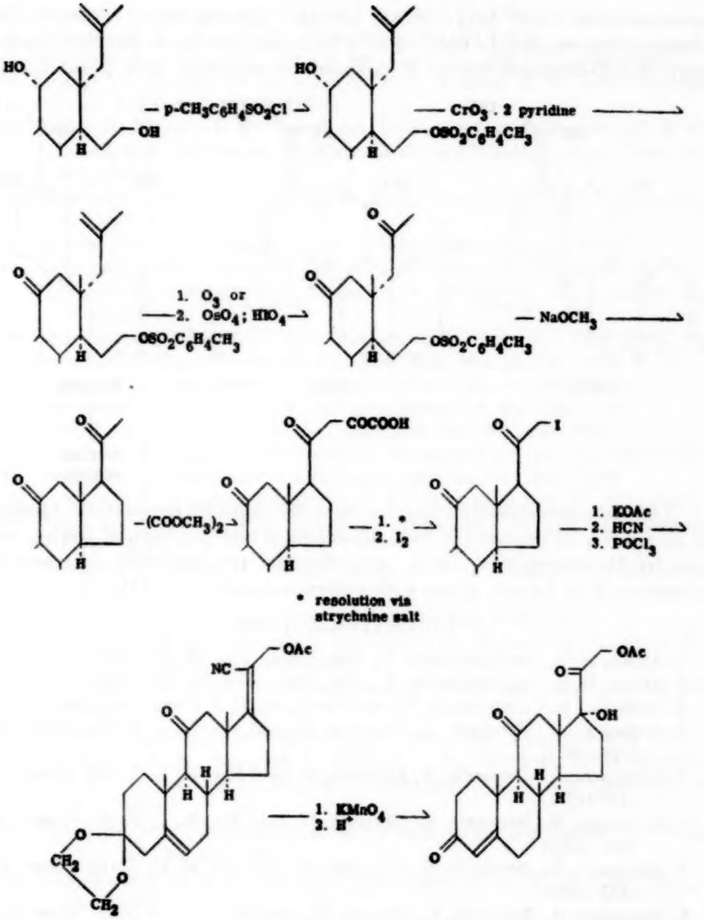
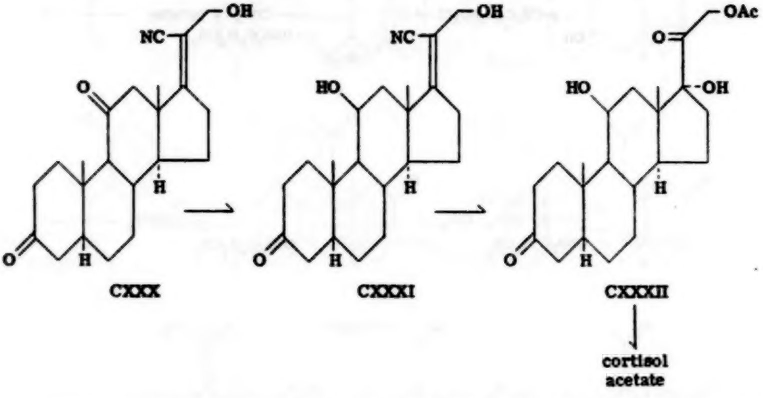


FIG. 8 (continued)

substance which appears to possess therapeutic activity equivalent to that of cortisone. Two partial syntheses have been described by Tishler *et al.* (297, 298). 21-Hydroxy-3,11-diketo-20-cyanopregn-17-ene (CXXX) by reduction as the 3-diethylketal with lithium borohydride or sodium borohydride gives the 11β,21-diol (CXXXI), which may be hydroxylated with osmium tetroxide to 21-acetoxy-11β,17α-dihydroxypregnan-3,20-dione (CXXXII), and converted by the modified Mattox-Kendall method using

semicarbazide (133) into cortisol acetate. Alternatively, cortisone-3,20-disemicarbazone may be reduced with lithium borohydride and the resulting cortisol-3,20-disemicarbazone hydrolysed by exchange with pyruvic acid.



The 11α-epimeride of cortisol has been described by Rosenkranz, Djerassi *et al.* (241) who prepared it by both chemical and biochemical routes, and also by Hershberg *et al.* (106), who obtained the 11α-hydroxyl group by reduction of an 11-keto group with sodium-propanol (cf. 111).

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NUTRITION[1]

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Maternal Nutrition and Congenital Malformations

The literature on congenital malformations is so voluminous, that it was decided to limit this review on nutrition to publications that are concerned with such malformations. Some have been mentioned previously but it seems necessary to mention them again in order to have a more unified account. This review is primarily concerned with modifications of the embryo that are the consequence of inadequacy of the maternal diet.

Nutritional deficiencies.—It has been known for some time that congenital deformities can be produced by dietary deficiencies. However, the first example that led investigators to suppose that inadequate nutrition might be an important cause of defective offspring was probably the one described by Hale (47). Four sows that had received a ration deficient in vitamin A bore 42 pigs, and all of them were blind. Some had no eyes, some had one, and some had one large and one small eye. There were also other abnormalities, such as accessory ears, subcutaneous cysts, failure of kidneys to ascend from the embryonic position, harelip, cleft palate, and misshapen hind legs. A deficiency of vitamin A in the maternal diet will also produce defective rat embryos (4, 117). There is at least one report that rat embryos may be defective if the maternal diet is deficient in vitamin E [Callison & Keiles (20)]. The defects were small eyes, failure of eyelids to open, and opaque membranes behind the pupil. Harman & Warren (52) gave guinea pigs a diet that was deficient in ascorbic acid on the 21st day of pregnancy. The diameters of the placental discs were reduced in the experimental animals, and the embryos were subnormal in size at all stages. The development of various organs, and of cell structure, was retarded also. The epithelial lining of the intestines of experimental animals was sloughed off.

Some of the most notable contributions to the subject of this review were published by Warkany and collaborators (115). They reported that female rats which receive insufficient riboflavin bear a high proportion of young with shortening of the mandible, radius, ulna, tibia, fibula, and with syndactylism, cleft palate, a thin abdominal wall in the ventral midline, protrusion of abdominal viscera, and defective eyes. Warkany's report has been confirmed [Giroud & Boisselot (40)], and it has also been shown [Giroud *et al.* (42)] that the embryos may be malformed although the mother shows no signs of a riboflavin deficiency, and although her tissues contain 80 per cent of the normal amount of riboflavin. A deficiency of riboflavin in the ration of hens is followed by micromelia in chicks that hatch from their eggs [Romanoff & Bauernfeind (104)].

[1] The survey of the literature pertaining to this review was concluded in August, 1952.

Gross malformations have been observed in rat embryos when the diet of the mother was deficient in pantothenic acid [Lefebvres-Boisselot (73)]. The more conspicuous abnormalities were hydrocephalus, anophthalmia or microphthalmia, exencephaly, and edema. Richardson & Hogan (103) reported that young rats may develop hydrocephalus as a result of deficiencies in the maternal diet. It was shown later (57, 91, 92) that if the maternal diet were deficient in folic acid the incidence of hydrocephalus was under 2 per cent. If there was a marked deficiency of cobalamin (vitamin B_{12}) the incidence was approximately 20 per cent. There was a smaller number of other abnormalities such as spina bifida, small or missing eyes, harelip, cleft palate, short lower jaw, short or kinked tails, and extensive edema. The essentials of the Missouri report have been confirmed in another colony [Richardson & DeMottier (102)].

It is our experience that the incidence of embryonic malformations is low when the maternal diet is deficient in folic acid. However, if sulfa drugs are added to the diet [Giroud & Lefebvres-Boisselot (41)] and, especially if a folic acid antagonist is included [Evans *et al.* (34, 87)], a typical series of malformations is produced in rat embryos. A deficiency of folic acid in the ration of hens [Sunde *et al.* (112)] is reported to cause a high incidence of syndactyly, deformed upper mandibles, and parrot beak in embryos that hatch from their eggs. Malformed embryos may also be produced by the injection of 4-amino folic acid into incubating eggs (67). When hens consume a diet that is deficient in biotin [Couch *et al.* (25)] their progeny have parrot beaks and shortened bones of the skull, wings, and legs. When the diet is deficient in cobalamin [Olcese *et al.* (93)] 80 per cent of the embryos die with myoatrophy of the leg, malposition, hemorrhage in the body, and perosis.

Female rats consumed a diet that contained 0.2 per cent of tryptophan and 10 mg. per cent of niacin [Pike (98)], and their offspring were normal. However, if the diet contained 0.2 per cent tryptophan and no niacin, or 10 mg. per cent of niacin and as small a quantity of tryptophan as 0.025 per cent, there was a high incidence of cataracts in their offspring.

Congenital malformations have been described which presumably are due to a deficiency of a nutrient that has not been identified with certainty. Sows consumed a ration made up essentially of yellow corn, soybean oil meal, alfalfa meal, calcium carbonate, and sodium chloride, and over 75 per cent of the pigs borne by second generation sows were abnormal [Ross *et al.* (106)]. The abnormalities mentioned included fused toes, club feet, paralysis agitans (with rotary oscillation of the head), vestigial limbs, kinked tail, and increased or decreased number of toes or dew claws, and either atrophied or hypertrophied eyes. Hart and co-workers (53) described calves that had an achondroplasia-like condition with shortened heads, frequently accompanied by an undershot jaw; usually the humerus and femur were much shorter than normal. The authors concluded that the condition was due to inadequate maternal nutrition between the third and sixth month of gestation.

Another example [Gillman *et al.* (39)] suggests a vitamin inhibition. Fe-

male rats were given subcutaneous injections of trypan blue and a high incidence, 19.2 per cent, of malformations could be detected at, or shortly after birth. The most common malformations, in decreasing order, were hydrocephalus, spina bifida, tail defects, and eye defects. Mice were affected in a similar manner (51).

Examples of mineral deficiencies that cause embryonic abnormalities are: iodine (109), manganese (10, 77), and copper (62).

It seems plausible that anoxia of the fetus may be due indirectly to inadequate maternal nutrition. Anemia in the mother, or difficult labor as a consequence of a malformed pelvis, would be a sufficient explanation. Windle (119) produced partial asphyxiation of guinea pig fetuses shortly before the normal time for delivery, and the survivors had characteristic neurological symptoms. Windle suggested that the human infant may be asphyxiated at birth, and though it has no detectable symptoms of brain damage, be mentally retarded. Pregnant mice were subjected to low atmospheric pressures, and there was a large increase in the number of fetal malformations [Ingalls, Curley & Prindle (61)]. The malformations included cleft palate, anencephaly, and an elongated head, but they ranged from placental remnants up to gross monstrosities.

There are also reports that anoxia may have unfortunate consequences for the human infant. In a study of the birth records of children with neurological symptoms, Schreiber (107) found a history of apnea in approximately 70 per cent of the cases, and he was of the opinion that cerebral damage was a consequence of apnea. It seems possible, however, that the apnea may have been a consequence of cerebral damage. Preston (100) investigated 132 cases of anoxia in human infants and found damage to the nervous system in every case. Ninety-seven were of normal intelligence and 35 were subnormal. Another study [Keith & Norval (68)] seemed to show that both prolonged labor and asphyxia are associated with abnormalities in infants, but apparently the survivors were not impaired mentally.

Physiological stress not related to inadequate nutrition.—Some of the other types of physiological stress reported to cause congenital malformations include: Injection of insulin (72, 74), cortisone (38), Compound F (66), and thyroxin (43); exposure to influenza-A virus (50), Newcastle disease virus (13), tetanus toxin (45), nitrogen mustards (55), sulfa drugs (2), physostigmine (3), selenium (99, 105), and irradiation with radium or x-rays (85, 118).

Undeterminant abnormalities.—The malformations that have been described were the consequence of an environment that was unsuitable for the embryo. It is commonly supposed that the malformations which occur in animals under field conditions are an inevitable result of the genetic inheritance, but in some instances the supposition may be erroneous. If so it is usually impossible to decide how a specific malformation should be classified. The examples described in pigs have included the following: hernia, cryptorchidism, and cleft palate (81); lobed ears, and deformed hind quarters (1); kinked tails (88); and defective skulls (58) in which the parietal bones did

not make contact along the median line. Blunn & Hughes (15) described external hydrocephalus and other abnormalities in pigs, which they thought were caused by a single recessive gene. Cole & Moore (23) described cases of hydrocephalus in calves when a certain bull was mated with his daughters; the herd ration was presumably adequate. Wright (123) developed a strain of polydactylous guinea pigs. The homozygotes were monsters which usually died in an early embryonic state, but a few survived to full term. Their legs were short and distorted and the feet were paddle shaped with from 7 to 12 short undifferentiated digits. The heads were abnormal, with a bulging forehead, and occasionally the brain protruded. Microphthalmia was invariable, and in some a cleft connected the nostrils and mouth.

One group of investigators has emphasized the importance of nongenetic factors in the development of the abnormalities they reported. Dunn *et al.* (29) concluded that the taillessness in rats, which they observed, was not determined by any single hereditary factor but was the consequence of some type of environmental stress. The degree of stress necessary to produce the abnormality was determined by the genetic constitution. A few years later these workers (44) described hereditary harelip in the house mouse, associated with cleft palate, pug-nose, and other abnormal structures. They were of the opinion that a combination of genetic factors and "a peculiar set of conditions" were responsible for the malformations. The food supply could easily be the peculiar set of conditions, but the authors made no mention of the diet.

There are numerous examples of micromelia in mammals, such as Dexter cattle, Dachshunds, and Ancon sheep; similar abnormalities have been reported frequently in birds. A strain of chickens was described [Asmundson (7)] in which the femur, tibia, and tarsometatarsus were less than half the normal length but were much thicker than is normal. Landauer (71) described creeper chickens in which the condition is a Mendelian dominant. This is presumably a lethal mutation, for homozygous creepers do not exist.

Incidence of malformations in man.—It is highly important to know how frequently congenital malformations occur in man, but there are wide differences of opinion on this subject. Some investigators believe the incidence of abnormalities is underrated (75), and there is a lack of agreement in the reports from different areas.

Witschi (120) states in his review of the literature that in many mammalian species, including man, one-third or more of the discharged ova are abnormal. Mall (78) estimated that in 100 pregnancies there are 20 abortions, and 2 malformed embryos. The incidence of malformations in the full-term infants is 0.625 per cent, and 7.5 per cent in aborted embryos. One would not expect Mall's method of collecting embryos to give a representative sample, but his estimate is in excellent agreement with some of the recent studies, and it is probably approximately correct. In the year 1944 (114) there was a total of 60 deaths with congenital malformations in the United States for

every 10,000 births. Of this number 51 were under one year of age. Murphy (86) reported that, according to the Bureau of Vital Statistics of the State Department of Health, Pennsylvania, approximately 80 infants of each 10,000 infants born in Philadelphia during the period 1929 to 1933, died with a congenital malformation. However, of these 80 the diagnosis was not confirmed in 32 cases by an independent investigation, and these 32 were discarded from Murphy's list. There remained then, 48 confirmed diagnoses. Presumably this is a minimum figure for in all probability some of the 32 that were rejected were actually malformed although there were no recorded observations to confirm the diagnosis. Furthermore, one would suppose that some malformed infants had survived and, therefore, were not recorded as abnormal. The central nervous system was affected in 60 per cent of the malformations, and the most common abnormalities were hydrocephalus, spina bifida, and anencephalus.

According to the birth certificates issued in New York State, outside of New York City during the period 1940 to 1942 [DePorte & Parkhurst (27)], 1 per cent of all infants born had a congenital malformation. The percentage distribution of the more important malformations was: extremities 27; central nervous system 22; harelip and cleft palate 9; heart 4. A comparison of the reported cause of death with the information recorded on the birth certificate, showed that 45 per cent of the malformations were not recognized at birth. However, a considerable number of babies that survived may have had a disabling malformation, and it was estimated that half the total malformations were not reported. At that rate the incidence of all malformations would be 2 per cent. McKeown & Record (80) stated that about 70 per cent of the malformations in still births, and 40 per cent of the malformations in first year deaths involve the central nervous system.

The records of maternity hospitals have supplied a large amount of data on the incidence of congenital malformations, but unless one has on-the-ground familiarity with the hospital policy there is always the suspicion that the patients come from an unrepresentative segment of the population. However, several selected examples are given in brief form to show that congenital malformations are much more common than most of us have supposed. Malpas (79) made a study of 13,964 consecutive births in the Liverpool Maternity Hospital during the years 1923 to 1932 inclusive, and estimated that 2.1 per cent of the infants had serious congenital defects. One-half of these involved the central nervous system. Javert & Stander (63) state that in their clinic in the New York Hospital, 27,000 infants had been delivered, and that 2.95 per cent had a malformation. They mentioned birth marks, extra digits, umbilical hernia, spina bifida, hydrocephalus, and cryptorchidism. Drillien (28) reported that in Simpson Memorial Pavilion, Edinburgh, for the three year period 1943 to 1945 inclusive, the incidence of fatal malformations among the infants was 1.47 per cent. Greenhill (46) reported that in the 2 year period from July 1, 1944 to July 1, 1946 the infant mortality rate in the Chicago Lying-In Hospital, from malformations, was

0.37 per cent. The number of malformed children that survived was not given.

According to Stevenson, Worcester & Rice (111) the percentage of malformations in infants born in the Boston Lying-In Hospital through the years 1930 to 1941 was 2.3; most of the malformations developed within the first 3 months of pregnancy. Miller (82) reported that 1.6 per cent of the infants born in the University of Kansas Medical Center had a malformation. Carter (21) examined the records of a group of mothers who were confined at Queen Charlotte's Hospital in the period between January 1943 and July 1949, and estimated that 1.47 per cent of the fetuses were malformed. The central nervous system was involved in over 40 per cent of these cases. In a subsequent publication Carter (22) estimated that about 2 per cent of new born babies will have a congenital malformation. He expressed the opinion that achondroplasia is entirely determined genetically and is due to a single dominant genetic factor. Stark (110) reported that in the General Hospital, South Shields, England, the incidence of fetal abnormalities was 1.39 per cent.

Disease and malformations.—It seems plausible that disease in the mother could cause malformation in the embryo, but apparently there is only one authentic example, rubella (8, 113). Carter (22) thought rubella could account for only a small proportion of the total number of malformations, and there is little or no evidence that congenital malformations are a consequence of any other disease. Hartman & Kennedy (54) reported that the incidence of congenital malformations in a series of infants which they studied was the same in mothers that had been ill in the first trimester of pregnancy as it was in those with no history of illnesses.

Nutrition and congenital malformations in the human infant.—It is well known that malformations in animals can be produced by dietary deficiencies and several investigators have studied the relations between the diet of the human mother during pregnancy and the well-being of her infant. Ebbs, Tisdall & Scott (30) selected women for their study who had not been pregnant over 6 months, who expected to be confined in the Toronto General Hospital, and whose income was low and whose diet was poor. Each alternate patient was given a daily supplement of protective foods. The women who received food supplements, and their infants, were superior to the test group in physical fitness, but there was no mention of congenital malformations. A group of investigators at Harvard University [Burke *et al.* (18, 19)] made similar observations and came to about the same conclusion, though they observed congenital malformations; thirty one mothers received a good or excellent diet and one baby had a defect of the heart; thirty six mothers consumed a diet that was poor or very poor, and eight of the infants had abnormalities; the abnormalities included cleft palate, hydrocephalus, cataract, mental retardation, and heart disease. Worcester, Stevenson & Rice (122) found that a considerable proportion of the mothers of malformed children were anemic. The maternal hemoglobin level was less than 75 per

cent in 39.4 per cent of the mothers and less than 65 per cent in 12.6 per cent.

There is some evidence that during the Second World War there was an increase in the incidence of malformations in German children. According to one report (90) the percentage of abnormal births in the city clinic for women in Chemnitz was 1.15 in the years 1931 to 1933, and in the years 1945 to 1949 it was 2.40 with a high of 3.44 per cent in the final year. The incidence of malformations was highest when the mothers were in the younger age groups, and was higher in the first-born than in children born subsequently. Data obtained in the clinic for women at the University of Leipzig (6) show that in the years 1936 to 1939 the percentage of congenital malformations was approximately 0.75, and during the years 1940 to 1946 it was over 1, with a maximum of 1.27 in 1943. There was a decrease after the war, to 0.33 per cent in 1948. Malformations of the central nervous system accounted for about 30 per cent of the total number. However, in the years 1945 to 1948 there was a sharp rise in the proportion of malformations of the central nervous system. These German investigators ascribed the high incidence of congenital malformations in the war years to malnutrition, an explanation that seems reasonable.

In England and Wales the rate of infant mortality fell during the war years [Logan (76)]. The high point was in the years 1940 to 1942, but there was a decline in the following years to a low of 0.445 per cent in 1948. There was an almost identical decline in the total infant mortality rate during that period. In the early years of the war the British Government took measures to improve the quality of the diet of the lower ecnomic group. It seems plausible that an improvement in nutritional state could reduce both the incidence of malformations and the infant mortality rate.

Smith (108) reported some of the effects of an acute food shortage upon the population of Holland. The percentage of congenital malformations in the prewar years, in Rotterdam and The Hague, was 1.36. This percentage did not rise in infants born during the period of acute hunger, but it rose in those infants, born after the hunger period, who were in the stage of differentiation during the period of acute hunger. In the last three months of 1945 the incidence of congenital malformations was 2.95 per cent or more than twice the reported incidence in any preceding period. The difference did not have statistical significance because of the small number of births studied, a total of 182.

Admittedly, the examples just cited are mostly historical observations rather than experimental researches, and there is no certainty that nutritional status was the only variable. One report has come to our attention of a nutritional deficiency that was not associated with congenital malformations. Brzezinski, Bromberg & Braun (17) reported that a group of 120 mothers in Palestine with riboflavin deficiency did not bear a single malformed child. The weakness in the report is the great uncertainty which prevails as to the reliability of the criteria for a deficiency of riboflavin in man.

A rating of the adequacy of a diet is difficult, expensive, and time consuming, and some investigators have substituted economic rating for nutritional rating. An economic rating is usually objective, and, presumably, poverty and malnutrition are associated. However, the reports on this topic are conflicting and leave the question undecided. Baird (9) compared the incidence of deformities in infants from the upper and from the lower economic and social classes in Aberdeen; supposedly the standard of medical care was about the same in the two groups studied. The incidence of fatal malformations in the less prosperous group was 0.72 per cent, and in the more prosperous group, 0.35 per cent. Baird thought the higher incidence of malformation in the less prosperous group was a consequence of poor health and nutrition.

It should be emphasized, though, that a large majority of those who have described congenital malformations have ascribed little or no importance to maternal nutrition. As a rule, nutrition of the mother is not mentioned. Murphy (86) estimated that over 60 per cent of the 571 defect-bearing families he studied were poor or very poor, and 3 per cent were well-to-do. In a hospital in which most of the patients were women of the lowest economic status, there were 113 malformed infants per 10,000 live births, which was more than twice the rate for the entire city. This suggests some correlation between poverty and congenital malformations, although Murphy himself thought the correlation had little significance. He did state, however, that the mothers of the malformed infants whom he investigated consumed diets that were far below desirable standards. Woolf (121) reported that in the five year period 1934 to 1938 the average infant mortality rate in England and Wales, ascribed to congenital malformations, was approximately 0.6 per cent. The rate was practically the same in the highest and lowest economic and social groups. Carter (22) was of the opinion that malnutrition is not correlated with malformations of the young. He stated that malformations were not more numerous in the lowest social and economic class than at the higher social and economic levels.

Complications of pregnancy and malformations.—Malpas (79) noted a higher incidence of abortions, stillbirths, and neonatal deaths among normally formed siblings of infants with malformations than is found in the general population. Kotz, Parker & Kaufman (69) estimated that 4.9 per cent of the infants had congenital malformations when the mothers were threatened with abortion. As is shown in Table I early infant mortality is associated with a high incidence of congenital malformations.

TABLE I

INCIDENCE OF MALFORMATIONS

Still births	Neonatal deaths	Survivors	
per cent	*per cent*	*per cent*	
6.25	22.1	1.19	Miller (82)
15.9	13.2	1.7	Stevenson *et al.* (111)

It is our impression that at one time this high incidence of malformations would have been regarded in most cases as merely a lethal mutation. It would seem equally plausible though to assume that in some cases malformations, inability to survive, and abortions are all the result of an unfavorable factor. As has been pointed out elsewhere, there is some evidence that inadequate maternal nutrition may be such a factor. Needless to say though, other plausible explanations have been suggested. It was stated in an unusually stimulating publication [Witschi (120)] that overripeness of the amphibian ovum produces some of the malformations, teratomas, and cancerous foci observed in the embryo. It also produces sex reversal and partial or complete sterility. It would be desirable to know whether or not this observation has any application to mammals.

Age of mother and congenital malformations.—It seems certain that mongolism is more frequent when the mothers are of advanced maternal age (14, 21, 26, 61), but there is a difference of opinion as to the correlation between incidence of other abnormalities and age of the mother. Malpas (79) reported that anencephalus, hydrocephalus, mongolian idiocy, and congenital cardiac disease occur more frequently in the infants of the older mothers, but he was uncertain as to whether there is any correlation between age of mothers and the occurrence of other malformations. The incidence of still births from fetal malformations in Scotland [Woolf (121)] during the year 1939 was 0.4 per cent for mothers under 20 years of age, and 0.95 per cent when the mothers were 40 years of age or more. According to Drillien (28) the incidence of fatal congenital defects is higher in multiparae than in primaparae and is higher in mothers that are 30 or over than in mothers that are under 30. Murphy (86) found the lowest proportion of malformed to normal infants in mothers aged 15 to 29 years. After age 30, however, the proportion of malformed children rose sharply. Carter (21) and Worcester, Stevenson & Price (122) disagree with Murphy, and are of the opinion that the alleged rise in the incidence of congenital malformations with age of the mother (as reported by Murphy) may be the result of an unsuitable statistical method.

Recurrence of malformations in siblings.—Malpas (79) reported that following the birth of one malformed child the incidence of malformations of the cerebrospinal system in subsequent children was approximately 2 per cent, about twice the number that was found in the general population. According to Record & McKeown (101) the incidence of congenital malformations of the central nervous system was about 0.45 per cent. However, when these women with deformed infants bore subsequent children the incidence of this malformation was 2.77 per cent. Carter (21) made a similar report, but the number of observations was too small for the difference to have statistical significance.

Of the women, studied by Murphy (86), who bore malformed infants there were 275 that bore at least one subsequent child. The total number of live births was 383, and 11.2 per cent of these were defective. The rate in the general population is 0.47 per cent, or 1/24th the rate in families that already

have one malformed child. This led Murphy to the conclusion that the malformations are genetic in origin. From one point of view it is correct to say that all malformations are genetic in origin. However, one would not expect the environment of the families to change much. If the environment had a causal relation to the first malformation one would expect it to be operative in subsequent births.

Sex and incidence of malformations.—Murphy (86) estimated that in the anencephalics there are about three females to one male. However, there are more males than females that have hydrocephalus, pyloric stenosis, harelip, and cleft palate. Stevenson, Worcester, & Rice (111) agreed with Murphy that there are three times as many females as males with anencephalus, but were of the opinion that this was the only one of the malformations that was related to sex.

Mental defects.—A question of considerable practical importance is whether mental defects originate in the same way as do physical defects. One would suppose they do, for it seems plausible that a defect in the structure of the brain could be followed by a defect in its functional activity. Penrose (97) estimated that the incidence of mongolism at birth is approximately 0.143 per cent. Ingalls (60) cited an estimate of 0.34 per cent by another worker. There are several reports of extensive deposits of lipids in the nerve cells, and some regard mongolism as a consequence of lipidosis (49, 64, 89).

Children in institutions for the feeble-minded were classified by the economic and social status of the homes from which they came [Halperin (48)], and there was no correlation between the classification of the home and the incidence of idiots or imbeciles. However, three-fourths of the morons were from below-average homes. Two-thirds of the borderline cases were from this group, but the total number in this classification was only nine.

Ingalls (59) reported that mongolism is accompanied by numerous malformations of the bones, such as syndactylism, and failure of metacarpals and phalanges to develop. Other malformations were described in the eyes, ears, heart and blood vessels, kidneys and endocrine organs. Hydrocephalus, spina bifida, cleft palate, and harelip were also observed.

Nutrition and undeterminant malformations.—Up to the present the reviewer has tried to emphasize well documented facts, with relatively little interpretation. From this point on, however, interpretative or speculative aspects will be emphasized. The reviewer's environment has led him to assume that environmental factors are important in the development of congenital malformations, both in animals and in man. However, regardless of the form in which the following pages are cast, the suggestions made should be regarded as questions rather than as opinions.

The first question is concerned with undeterminant congenital malformations in animals. Is there any reason to suppose that the rations of the females that bore abnormal offspring were inadequate in any degree? There is every reason to suppose that they were. Several of the important recent discoveries

in nutrition had their origin in the knowledge that the commonly used rations of poultry and swine had been partially inadequate. One without experience might think it to be a simple matter to formulate a ration of common commercial feedstuffs that would be, by itself, entirely adequate; but this is not the case. When experimental animals in the past had no opportunity to select their food, and received no food except that which was supplied to them, rations made up of the commonly used commercial constituents were usually inadequate in some degree. Undoubtedly many rations in use now are slightly deficient in some respect, and before 1940 most of them were probably deficient in several respects. A few examples of improved growth rates are included as illustrations. Osborne & Mendel (94) reported in 1912 that young rats gained at the rate of about 15 grams per week. Anderson & Smith (5) reported 20 years later that rats would gain nearly three times that fast. It is common knowledge that poultry and swine will gain 50 per cent faster now than they did 30 years ago. The improvement in the adequacy of livestock rations during gestation and lactation is especially striking. There are authentic reports that brood sows on rations which more than met the nutritional standards of 20 years ago have lost two-thirds or more of their pigs during lactation, and the weaning weight of the survivors was two-thirds or less of the normal weight. We have observed sows that were fed according to current standards; they weaned over 95 per cent of their pigs, and the weaning weight was 50 per cent higher than was normal according to earlier standards. The observations of Ross and collaborators (106) on congenital malformations in new-born pigs add support to the conclusion that a ration could meet the nutritional standard of 10 years ago and still be grossly inadequate. It may be then that one cause of undeterminant congenital malformations in animals is an inadequate maternal diet.

Another possible cause is an unfortunate genetic inheritance that makes an animal require more of a nutrient, such as a vitamin, than is required by the great majority of the population. Some of the examples are shown in Table II.

TABLE II

DIFFERENCES IN NUTRIENT REQUIREMENTS

Animal	Nutrient required in different amounts by different strains	Citation
Chick	Riboflavin	Lamoreux & Hutt (70)
Mouse	Riboflavin	Fenton & Cowgill (36)
Mouse	Pantothenic acid	Fenton *et al.* (37)
Mouse	Protein	Fenton & Carr (35)
Rat	Choline	Copeland (24)

There are also strain differences in resistance to toxic substances. One well-known example is varying susceptibility of rats to cataract when they consume excessive amounts of galactose or lactose [Ershoff & Deuel (33);

Mitchell (84)]. There are also several examples of the effect of environmental temperature on vitamin requirements, as presented in Table III.

TABLE III

Environmental Temperature Dependence of Vitamin Requirements

Animal	Nutrient required in different amounts	Citation
Rat	Vitamin A	Ershoff (31)
Rat	Thiamine	Ershoff (32)
Rat	Thiamine	Hegsted & McPhee (56)
Pig	Riboflavin	Mitchell *et al.* (83)

The second question is concerned with congenital malformations in man and the probability that they are a consequence of inadequate maternal nutrition. Satisfactory evidence is not available, and one can only form an opinion as to the probabilities. It is the opinion of the reviewer that the data previously cited lend plausibility to the hypothesis that congenital abnormalities and nutrition are interrelated. It is frequently stated and it may be true that no nation in history was as well fed as is the United States today, and the average American diet is probably not seriously deficient by any reasonable standard. However, if there is an average diet there is also a below-average diet, but we do not know what proportion is low enough to produce a detectable effect. Furthermore if there is an average requirement there must also be an above-average requirement, and we do not know in how many people the excess requirement is high enough to produce a detectable effect if this excess requirement is not satisfied. There are few data on unusually high requirements, but it has been known for some time that there are cases of infantile rickets that do not respond to the usual dosage of vitamin D but will respond to massive doses [Pedersen & McCarroll (96)]. The evidence supplied by comparative physiology leads us to believe that this is an isolated example of a common phenomenon.

It should be emphasized though that some of the data are not in harmony with our suggestion. For example, if our hypothesis is correct one would expect a high incidence of congenital malformations in poverty stricken, poorly-fed populations, and reports of this kind have not come to our attention. The only data that might have a bearing on this point (114) were obtained in the United States or from territory under its jurisdiction. The total number of deaths with congenital malformations per 10,000 live births, in 1944 were: United States—white 63, negro 35; Puerto Rico—white 27, nonwhite 8; Hawaii—all races 36; Virgin Islands—all races 47. If these data are accepted one would probably conclude that a good diet increases the incidence of congenital malformations. A more probable explanation is that a higher economic and educational level increases the accuracy with which causes of death are reported.

If a significant number of congenital abnormalities is to be produced

in animals by supplying the mothers with deficient diets, it is necessary that the deficiency be severe. It is the common view that this degree of severity does not occur in human diets, and that congenital malformations in man are seldom, if ever, a consequence of a nutritional deficiency. As indicated previously, this view would probably be correct if all mothers consumed an average diet and had average nutritional requirements. Individuals that develop abnormalities then, as a consequence of nutritional deficiencies, may be divided into at least two groups, though there is no evidence as to the total or relative numbers: (*a*) those individuals having a normal requirement for a nutrient, riboflavin (for example), and never developing an abnormality unless the supply is low; (*b*) those individuals having an abnormally high requirement for riboflavin and possibly developing an abnormality even though by ordinary standards the supply of the vitamin is adequate. Our knowledge is too fragmentary to explain the nature of the fault, but a hypothetical example may convey the type of explanation we have in mind. One might suppose that an embryo would begin to develop abnormally at a certain stage if some specific enzyme were present in too small a quantity; for example, X+riboflavin+phosphate→enzyme. If the reaction to the right proceeded readily, the embryo would never become abnormal unless the supply of riboflavin were inadequate. If the reaction did not proceed readily, a normal supply of riboflavin might yield an insufficient amount of the enzyme, and the embryo would become abnormal unless the supply of riboflavin were made unusually liberal. If the reaction proceeded too slowly under any conditions, the abnormality would be inevitable in any environment. Some such irregularity, or variability in metabolism, would explain all of the more common malformations. One might suppose, for example, that a deficiency of cobalamin in the human diet would be exceedingly rare. However, if taken by mouth, the patient with pernicious anemia must consume an enormous quantity, and it is reasonable to suppose that for such a person many diets are deficient in cobalamin. It seems highly probable that pernicious anemia is not an all or none affair, but that there are all grades, intermediate between the extremes of severe disease and absence of any symptom of disease. One would expect the intermediate cases to be more numerous than those that are detected. If an intermediate case became a mother it seems possible that for her the average diet would be seriously deficient in cobalamin, and that an infant she bore would have the disabilities that are a consequence of that deficiency. There are numerous examples of biochemical abnormalities.

The hypothesis just suggested may help to explain how the same abnormality may be produced by several different methods. On reading the literature one is impressed by the monotonous regularity with which the same abnormalities are described over and over. A plausible explanation is that they are all produced by the same block in the biochemical mechanism. X-rays may destroy a gene that determines the course of a biochemical reaction. Some substance such as selenium, nitrogen mustard, or tetanus toxin, may

inhibit or destroy an essential component of the mechanism. Though the path of interference is different the result is the same.

To return to the possible responsibility of nutrition for malformations there are probably no quantitative assays of the poorest diets that are consumed in the United States, which may conceivably make up 5 or 10 per cent or more of all the diets. One could expect them, however, to be in some respects lower than any recommended allowance. This is partly due to the fact that as the income declines the proportion of the population that has ready access to the protective foods, meat, milk, eggs, and leafy vegetables, decreases. These protective foods as a rule are the most expensive and are the first to be eliminated when the budget for food is limited. If one were to seek unambiguous evidence for the point of view just mentioned, the reviewer would not know where to find it, but it has some degree of probability. The supporting facts that come to mind are: (*a*) practically every malformation described in man can be duplicated in animals by experimental nutritional deficiencies; (*b*) it seems probable that there is a higher proportion of congenital debility and of malformations among individuals in the lower economic and presumably lower nutritional levels; (*c*) there is a higher incidence of some malformations among the younger children in a family than in the older. Such a distribution would not be expected if only genetic factors were concerned with the abnormalities. It is true that if a woman bears one malformed infant there is increased probability that subsequent children will also be malformed. This distribution would be expected from the facts of heredity, but it would also be expected from the facts of nutrition. Food habits are not easily changed, especially when there is no change in economic status. Furthermore, it is easy to suppose that a family may increase in size faster than does the income which supports it, and that the family diet becomes less adequate as the number of children increases. It is also easy to believe that if the protective foods become scarcer as the family becomes larger, a mother may deny herself in order to protect the child she has now and thus do irreparable damage to the child she will have in the future.

To return to the relative importance of genetic and nutritional factors in the development of malformations, it seems to the reviewer that the responsibility cannot be divided. Under some circumstances the nutritional status of an animal determines what will happen with a given genetic background. Conversely the genetic background determines what will happen with a given nutritional status. Two papers will be mentioned as having some bearing on this point, though the authors may disagree with the reviewer. Kallman, Barrera & Metzger (65) described a family of eight children, four of whom were afflicted with bilateral anophthalmos. Two of the four were identical twins and were also mentally retarded. The authors were of the opinion that the abnormality was associated with the genetic inheritance, which made the embryo abnormally sensitive to some unknown nongenetic influence. If this suggestion is correct, a nutritional abnormality is as plausible an explanation as any other. Presumably the abnormality would be un-

usual sensitivity to a mild nutritional deficiency or an unusually high requirement for one or more nutrients. Böök & Rayner (16) analyzed the data on anencephalus reported by the Departments of Obstetrics of the Universities of Lund and Malmo. They stated that their data suggested, but did not prove, a genetic explanation for the malformations. They found no evidence that any exogenous factor was involved, but there is no internal evidence that they looked for such a factor. Nothing was said about diet, or about social or economic status of the families. They did look for the possibility that disease was involved, but excluded the possibility.

Problems.—The chief object of the preceding pages is to suggest that many malformations in infants may be preventable. However, the death rate among infants ascribed to congenital abnormalities is only about 0.5 per cent, and there may be a difference of opinion as to whether this should be a cause of concern. However, the known facts raise questions that should be considered.

(*a*) Are there mild cases of congenital abnormalities that are not readily diagnosed? If these mild cases do occur, what percentage of the total number of infants is affected? There is no answer to this question, but some of the facts observed in our rat colony may be worthy of consideration. In a group of 150 newborn animals whose mothers consumed a diet that was deficient in cobalamin, there were 72 hydrocephalics that could be diagnosed by inspection and 78 with no external symptoms of abnormality. The brains of 50 of the 78 were sectioned and microscopic examination revealed that 32 of them or 64 per cent were at least slightly abnormal.[2] Beckett, Netsky & Zimmerman (11) described eleven cases in man of stenosis of the aqueduct of Sylvius which they believed to be developmental in origin. Seven of the cases could not have been diagnosed at birth. The report gives no data on the incidence of this type of stenosis, but evidently it can occur with no external symptoms. A similar stenosis is observed in our rats with experimental hydrocephalus, and in addition the cell structure in the roof of the cerebral aqueduct is abnormal [Overholser *et al.* (95)].

(*b*) Is there any relation between maternal nutrition and the functional activity of the brain of the progeny? A study has been carried out in our laboratory in an attempt to answer that question [Whitley, O'Dell & Hogan (116)]. Female rats received a diet which was believed to be practically devoid of folic acid and was probably partly deficient in cobalamin. On this diet less than 2 per cent of the young were hydrocephalics, and the young that were used in the investigation had no symptoms of hydrocephalus. The experimental and control rats were evenly matched in sex, age, and weight, and the test of learning ability was the number of trials required to learn the way through a water maze, similar to the one used by Bernhardt (12). The average number of trials required by the controls to learn the maze was 14.1, and by the experimental animals, 21.7. The statistical evidence of signifi-

[2] Data supplied by J. R. Whitley and B. L. O'Dell (unpublished).

cance was overwhelming. The author is convinced that the functional activity of the central nervous system in rats can be impaired by some type of inadequacy in the maternal diet. To the author it seems significant that although only 2 per cent of the young had detectable hydrocephalus, most of them had a functional impairment of the central nervous system. Whatever the facts may be in our rats, though, the application of these observations to man is in the realm of speculation. One would suppose that, as a rule, members of the lowest economic groups consume diets that are less adequate than the diets consumed by groups with larger incomes, but the effect on the intelligence ratings of the children is controversial. It is a common opinion though that the average of intelligence in poverty-stricken families is below that of children from families that can afford a more adequate diet, and that, as a rule, the very poor are poor because they are stupid. If there is any correlation between economic level and intelligence, it may be that cause and effect are reversed in public opinion, and that some of the stupid are stupid because they are poor.

We would like to start the next generation out with a higher physical and mental standard than this one could attain, but unfortunately it may be some time before we can be sure that we know how to do it. It seems to the reviewer that the first thing to try is an improvement in the food supply, at least for those who are not well fed now. Food quality is important for the entire population but is of most critical importance for mothers. It is important for them at all times but is of most critical importance in the period that begins shortly before the child-bearing age is reached, and ends not long after the child-bearing age is passed.

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THE CHEMISTRY OF NEOPLASTIC TISSUE[1,2]

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The application of biochemical and chemical methods to the problems associated with the induction, characteristics, and control of neoplastic cells continues to yield increasing amounts of new information. As a mere listing, without comment, of the publications on these problems in the past year would more than fill the space allotted to this review, only a small portion of the findings can be discussed here. For this reason, the review will be restricted to a discussion of results having a direct bearing on animal tumors; such areas as radiation effects, and immunology of tumors, recently reviewed by Hauschka (1), will not be covered. The authors take this opportunity to apologize for the necessary omission of many worthwhile studies and also to refer readers to the annual reports of the British Empire Cancer Campaign and the American Cancer Society for general summaries of the majority of the cancer research projects now under way.

In our survey, we have been particularly impressed with the accumulating evidence of the importance of endocrinological factors in the induction of cancer, and also by the striking differences among the various types and strains of tumors. Closer study of these differences may reveal the biochemical abnormalities most closely associated with the problems of abnormal growth.

Carcinogenesis

Boyland (2) has speculated that the carcinogenic process is initiated by effects on chromosome nucleic acids. He has suggested the following five types of reactions on desoxyribonucleic acid (DNA) to explain the action of known carcinogenic stimuli: depolymerization, precipitation, cross linking, complex formation, and inhibition of synthesis. At the moment, this attractive hypothesis suffers from the lack of direct supporting evidence, although it cannot be dismissed on that account. Such chemical evidence as has been obtained to date indicates that, *in vivo*, carcinogens are bound to protein rather than to DNA.[3]

The possible role played by protein-bound metabolites of azo dyes and hydrocarbons in carcinogenesis has recently been summarized by Miller &

[1] The survey of the literature pertaining to this review was concluded in November, 1952.

[2] The following abbreviations are used: AAF for acetylaminofluorene; DNA for desoxyribonucleic acid; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); PNA for pentosenucleic acid; HN_2 for nitrogen mustard [bis (β-chloroethyl) methylamine].

Miller (3). The rate at which the protein-bound azo dye in the liver reaches a maximum level has been found to be correlated with the carcinogenic activity of the dye. However, neither the actual level reached (higher levels are reached with two of the very weak carcinogens than with active ones) nor the rate nor extent of the decline in bound dye level is correlated with activity. The tumors produced contain no bound dye although at the time of their appearance the level of bound dye in the liver is 50 per cent or more of the maximum level attained. The bound dye data, *per se*, therefore may be interpreted to indicate either that a gradual depletion of a specific protein plays a causal role in carcinogenesis or that the enzyme systems involved in binding azo dyes are incidentally lost along with other specialized systems in hepatic cells when they become tumor cells. Perhaps, as more detailed information is obtained on the rates of loss of bound dye in different parts of the cell, clear-cut evidence indicating a relationship between the gradual deletion of a specific protein component and the formation of tumors will be provided.

On the quantum mechanical side, the hypothesis that the electron density in the K region of the hydrocarbon carcinogens is intimately concerned in the carcinogenic process continues to excite interest. Greenwood (4) has recalculated electron densities by the molecular orbital method for a series of 1,2-benzanthracene and benzacridine derivatives. The calculations for the same compounds are in good agreement with those obtained earlier by the valence bond method, but the results of the more extended series indicate that a correlation between variation of carcinogenic power and charge density of the K region does not hold in all cases. Kooyman & Heringa (5) suggest that difficulties of this sort may be resolved by the extension of the area of inspection to cover a larger portion of the molecule. Greenwood concludes that if the K region does play some role in the carcinogenic activity of these molecules then it most probably acts by an ionic rather than a free radical mechanism.

Carcinogenic agents.—In order to establish structure-activity relationships it is necessary that adequate data on biological activity be obtained. Comparatively few new compounds were reported in the last year to be carcinogenic. Steiner *et al.* (6) have reported that 1,2-benzanthracene, heretofore thought to be noncarcinogenic, possesses moderate potency when tested on the skin of C_{57} black mice. Among the aminofluorene derivatives, Bielchowsky & Bielchowsky (7) observed that N-dimethyl aminofluorene was less carcinogenic for rat liver than the N-methyl or primary amine but that the incidence of breast cancer was similar for all three. Likewise, Miller and associates (8) have found that while a variety of aminofluorene analogues produced mammary tumors, only those containing a —CH_2— bridge, or capable of being reduced to a —CH_2— bridge, possessed activity as liver carcinogens. In view of the earlier finding that the oxidation of 1,2,5,6-dibenzanthracene to phenolic derivatives eliminated its carcinogenic activity, it is of interest that two oxidation products of acetylaminofluorene (AAF), 2-acetylaminofluorenol and 2-acetylaminofluorenone, were active. Two

oxidation products of a different series, 3,3′-dihydroxybenzidine (9) and 2-amino-1-naphthol (10) have also been reported to have carcinogenic activity. For the latter compound it has been stated (11) that those species which excrete the largest quantity of this metabolite of 2-aminonaphthalene in the urine are most susceptible to the carcinogenic action of the parent amine. Three new azo dyes: 3′-methylaminoazobenzene, 3′-fluoroaminoazobenzene (12), and 4-dimethylaminoazobenzene-1-azo-1-naphthalene (13), have been found to possess weak carcinogenic activity. The 3′-methyl- was slightly more active than the 3′-fluoro- compound and was converted to the N-methyl derivative *in vivo* to a greater extent (12).

A number of other interesting observations have been made. Gorbman (14) has found that the production of pituitary tumors in mice by I^{131} is not due solely to the "destruction" of the thyroid, because pituitary tumors are not formed when the thyroids are "destroyed" with low doses of this agent in mice maintained on a Remington low-iodide diet. Klein (15) has reported that urethane, injected into pregnant mice, induced a high incidence of lung adenomas in the offspring even though they were delivered by caesarian operation one hour after the injection and nursed by an untreated mother. Oppenheimer, Oppenheimer & Stout (16) have observed that even after cellophane was washed with alcohol, in an attempt to remove possible adsorbed carcinogens, it induced tumors when implanted subcutaneously in the abdominal wall of rats and mice; polyethylene films were also active. The possible importance of the *Senecio* alkaloids in the poor diets of the Bantus as a causal agent in the induction of liver cancer in these people is emphasized by the increased incidence of liver tumors in rats fed this material (17).

One area in carcinogenesis that is deservedly receiving more attention is the environmental causation of human cancer. The reader is referred to the following reviews and papers for information [Hueper (18); Smith *et al.* (19); Aub *et al.* (20); Waller (21); Dawson (22); Goulden *et al.* (23)].

Metabolism of carcinogens.—The necessity for more complete information on the metabolism of carcinogens as an aid to elucidation of their mode of action is now generally accepted. In this field Cook & Schoental (24) have isolated two oxidation products of dibenzanthracene from rabbit feces as crystalline methoxy derivatives and Wiest & Heidelberger (25) have recovered C^{14}-dibenzanthracene, unchanged, from mouse skin *in vitro* under conditions in which benzpyrene was metabolized. An unusually complete accounting for the metabolism of C^{14}-labeled benzene in the rabbit has been reported by Parke & Williams (26); no radioactivity was found in the respiratory CO_2. Weisberger *et al.* (27) have found that in normal rats AAF[2] can be absorbed through the stomach and that at least four metabolites are excreted in the urine, one of them being the 7-OH derivative. In the dog, one per cent of the AAF administered in the diet was recovered from the urine as the 7-OH metabolite (28).

Gutman *et al.* (29) found elevated tissue levels of AAF in partially hepatectomized rats but normal levels in riboflavin-deficient animals. This obser-

vation is of interest as Engel *et al.* (30) observed no influence of riboflavin on liver tumor production by AAF, in contrast to its marked effect on azo dye carcinogenesis. Allison & Wase (31), however, found that in riboflavin deficiency a smaller percentage of excreted AAF metabolites was conjugated than in normal animals and that pantothenate increased the extent of conjugation when added to low-riboflavin diets. Mellors & Hlinka (32) have observed that a fluorescent metabolite of orally administered 2-aminonaphthylamine was present in bladder mucosal cells but not in the gastric mucosal cells. They present evidence that the metabolite (or the parent compound) can reach the bladder cells by a route other than via the kidneys and ureters.

Information on the metabolism of the azo dyes has been supplied by Miller *et al.* (33) who have found that the N-methyl group is incorporated into the beta carbon of serine and the methyl groups of choline to a small extent. In folic acid-deficient rats less appears in serine and choline and in respiratory CO_2. In cobalamin (vitamin B_{12}) deficiency less appears in choline, and in riboflavin deficiency more appears in respiratory CO_2. Presumably the N-methyl of the azo dyes gets into the serine and choline molecules after preliminary oxidation to formaldehyde and formate, although the possibility that some of the azo dye N-methyl carbons become N-methyl groups on choline via a series of transmethylation reactions has not been completely ruled out. MacDonald *et al.* (34) have shown that the metabolism of the N-methyl group of two weak azo carcinogens does not differ from the metabolism of this group on strong azo carcinogens. 4-Dimethylaminoazobenzene has been reported by Dinning *et al.* (35) to prevent leukopenia in rats on a methionine-deficient diet in the presence of cobalamin; this suggests utilization of the N-methyl carbon. Berenbom & White (36) labeled 4-dimethylaminoazobenzene with N^{15} in each of the three nitrogen positions separately and found that the two nitrogens of the diamine ring were retained in the liver to a significantly higher extent than the nitrogen of the aniline ring. Although no information is available as to the nature of the retained nitrogen, this is of interest in view of the earlier hypothesis (37) that inhibition of hepatic cell enzymes by the aromatic diamine portion of the ring might play an important part in the carcinogenic process.

Engel (38) reported that the AEC strain of rats is much more susceptible to the carcinogenic action of 4-dimethylaminoazobenzene than the Sprague-Dawley strain and that livers of the former strain metabolize azo dyes *in vitro* more slowly. The AEC rats also show a high incidence of liver tumor when maintained on choline-deficient diets (39). No significant difference in susceptibility to azo dye carcinogenesis among several other strains of rats was found by Clowes & Harris (40).

The lack of carcinogenic or carcinostatic activity of methylcarbamate, as compared with ethylcarbamate (urethane), is not because of its more rapid destruction or excretion since it is retained in the body longer than urethane [Boyland & Papadopoulos (41)].

Viruses as causative agents.—The mammary carcinoma virus of the mouse is one of the most extensively studied. The production of tumors by

this agent is affected by an inherited hormonal influence which is associated with the development of adrenal cortical hyperplasia in gonadectomized mice [Bittner (42)]. The spherical particles isolated by Graff, Heidelberger & Haagensen (43) from mouse milk transmit the disease in high dilution and are antigenically distinct from the normal proteins of mouse tissue or milk. A mammary tumor virus has also been found in the house mouse [Andervont (44)].

The Berry-Dedrich transformation of fibroma into myxoma in the rabbit has been reviewed by Smith (45). The two viruses are closely related serologically. Fibroma, which is benign and noncontagious, can be changed into myxoma, which is lethal and highly contagious, by mixing the active fibroma virus with a heated extract of myxoma tumor tissue. The heated extract is active even after deproteinization with chloroform and is not destroyed by treatment with alcohol; on the basis of this stability Smith suggests that the active agent may be DNA.[2]

By treating chickens with methylcholanthrene, Duran-Reynals (46) has activated a latent fowl pox infection which causes typical acute lesions containing the virus. Later, papillomas, angiomas, and squamous cell carcinomas appear which frequently contain active fowl pox virus. Whether the activation of the virus is directly involved in the process of carcinogenesis, or whether it is an event concomitant with carcinogenesis which may affect the morphology of these tumors, is discussed. Sanford *et al.* (47) have concluded that the chick fibroblasts are the cells specifically susceptible to the Rous sarcoma virus, since the virus lived for six and one half months in them in tissue culture, whereas it survived only for short periods in the other normal cells studied.

Recently, Stasney, Cantarow & Paschkis (48) succeeded in transmitting the Murphy rat lymphosarcoma by injection of chromatin fractions supposedly freed of whole cells by centrifugation. Microscopic examination of hundreds of smears and paraffin sections revealed no evidence of whole cells or nuclei. Although Klein (49) confirmed this work in every detail on mouse lymphosarcoma, he believes that the preparations probably did contain whole cells, since transmission experiments in hybrid mice showed that the tumor had the genetic constitution of the donor and not that of the recipient.

The findings of Gross (50), on the transmission of mouse leukemia by cell-free extracts, await confirmation in other laboratories. The subject has been critically reviewed by Furth (51).

Winzler (52) has found that the protein metabolism of mouse brain is distinctly altered by infection with encephalomyelitis virus and suggests that the transformation from the normal to the malignant state may be a similar phenomenon.

Factors Affecting Carcinogenesis

Nutrition.—A number of general papers on the influence of diet on cancer were prepared for the University of Texas symposium in April (53) by Hoch-

Ligeti, Kensler, Engel, King and Morris. Only the more recent studies will be mentioned here.

The use of semipurified diets, in contrast to stock diets, led to an increased incidence of liver tumors in rats fed AAF[2] [Engel *et al.* (54)] and to an increase in the incidence of spontaneous liver tumors in two strains of mice [Silverstone *et al.* (55)]. In the latter experiments, the incidence of breast cancer and benzpyrene-induced skin tumors was not significantly altered. The findings of Silverstone seem to be in harmony with the opinion that the effects of diet on the chemical induction of liver tumors cannot be explained solely on the basis of alterations in metabolism of the carcinogen. Ekman & Strombeck (56) have reported that the production of bladder lesions by aniline in rats is altered by diet and that those diets giving protection lead to an increased excretion of *p*-aminophenol.

Copper deficiency (57) and the feeding of thiouracil [Paschkis *et al.* (58); Clowes & Harris (40)] were reported to inhibit azo dye carcinogenesis. The Paschkis experiments apparently rule out the possibility that the thiouracil effect on tumor incidence is directly related to the thyroid hyperplasia produced, as it was reported that the tumor inhibiting effect was prevented by feeding uracil, whereas the effect on the thyroid was not. The observation of Day *et al.* (59) that cobalamin inhibits azo dye carcinogenesis was confirmed by Miller and associates (33). Studies on the effects of antimalarial compounds [Rumsfeld *et al.* (60)] and vitamin E [Swick & Baumann (61); Hove & Hardin (62)] have also been reported.

Boutwell & Rusch (63) have shown that restriction in feeding time, combined with a low-calorie diet, did not yield results on tumor incidence different from those obtained by calorie restriction alone.

Other carcinogens.—Richardson *et al.* (64) observed that the administration of methylcholanthrene inhibits azo dye carcinogenesis and produces adrenal cortical changes. Miller and associates (65, 66) have suggested that the effect of methylcholanthrene on azo dye carcinogenesis is due to an increased rate of azo dye metabolism. The influence of mixtures of carcinogens has been studied by several groups (67, 68, 69).

Hormones.—Korteweg & Thomas (70) reported some years ago that the production of tumors in mice by benzpyrene was markedly delayed but not prevented by hypophysectomy. Moon *et al.* (71) have extended these findings by using methylcholanthrene as the carcinogen in the rat. Griffin *et al.* (72) have supplied the additional information that azo dye carcinogenesis is prevented, or at least markedly retarded, by hypophysectomy in the rat. These results, coupled with the earlier reports that administered growth hormone produced tumors in intact rats, but not in hypophysectomized rats, indicate that pituitary function is important in the induction of tumors. Moon *et al.* (73), however, have not succeeded in producing tumors in intact mice by the administration of twenty times as much growth hormone preparation as was effective in eliciting tumors in rats. That it was active as growth hormone in mice was indicated by the weight gain observed in the females.

Another inhibition, presumably hormonal, was reported by Salzberg & Griffin (74) who found that azo dye carcinogenesis was partially prevented in rats made diabetic by the prior injection of alloxan.

The influence of sex hormones on the induction of certain types of tumors is emphasized by the reports that: (*a*) the incidence of breast cancer is greatly increased in certain strains of rats by the administration of estrogens (75); (*b*) the activity of methylcholanthrene on prostatic grafts is changed by both androgens and estrogens (76); (*c*) testosterone decreases the incidence of postirradiation lymphomas in mice (77); (*d*) estrogens appear to decrease and androgens to increase the incidence of spontaneous hepatomas in mice (78); and (*e*) the incidence of liver tumors in male rats is higher than in females when AAF is the carcinogen (79). Bielchowsky & Hall (80) have reported an interesting study on the effects of AAF in parabiotic female rats in which one of the pair was gonadectomized. Burrows & Horning (81) have comprehensively reviewed the field of estrogens and neoplasia.

The carcinogenic activity of dibenzanthracene in mice is reported to be reduced by thryoxine administration and the difference is attributed to an increased rate of metabolism of the carcinogen (82).

Biochemical Characteristics and Effects of Tumors

Enzymology.—Much of the effort in this field in the past year has been directed towards elucidating the nature of oxidative energy-producing reactions and the extent to which they are carried out *in vitro* and *in vivo* in tumor cells. Busch & Potter (83) extended their study of citric acid cycle inhibitors *in vivo* to malonate and found that succinate accumulated in the four tumors examined to a greater extent than in most normal tissues. In the Flexner-Jobling carcinoma, amino acid disappearance was concomitant with succinate accumulation. Wenner, Spirtes & Weinhouse (84) surveyed the enzymes of the citric acid cycle in several tumors and found most of them to be in the normal range or higher. Aconitase and oxaloacetic carboxylase were lower than in most normal tissues. The data obtained to date thus indicate that tumor cells contain the enzymic equipment and do utilize the citric acid cycle *in vivo*.

Wenner *et al.* (85, 86) have shown that mitochondrial preparations from several tumors all actively oxidize pyruvate, provided DPN[2] is added. Kielley (87) and Williams-Ashman & Kennedy (88) have found that mitochondrial preparations of tumors fortified with DPN possess active oxidative phosphorylating systems. The latter authors (88) have reported the uncoupling of oxidation and phosphorylation in the tumor, as in normal tissue preparations, by dinitrophenol. Siekevitz *et al.* (89) have shown that oxidative phosphorylation is maximal only when the balance between phosphate donors and phosphate acceptors is optimal. There is a marked difference in this respect between the two tumors studied. Weinhouse and associates (90) have reported on fatty acid oxidation by several tumors. A net production of acetoacetate was found in liver but not in hepatoma.

Chance & Castor (91), using spectrophotometric assay methods, found that the cytochrome-*c* content (relative to cytochromes-*a* and a_3) in several lines of mouse ascites tumors was abnormally high. Cytochrome-*b* was barely detectable. Lenta & Riehl (92) studied DPNH[2] oxidation in acid-treated tissue extracts and reported high diaphorase and cytochrome-*c* reductase levels and low cytochrome-*c* levels in tumors.

Dickens & Glock (93) report that hexose monophosphate and 6-phosphogluconate are actively oxidized by extracts from a rat liver tumor but not by extracts from the Rous fowl sarcoma. Warburg & Hiepler (94) found zymohexase activity in fresh ascites fluid from mice with Ehrlich mouse ascites tumor. The zymohexase activity of the cells remained constant for many hours under aerobic conditions but increased when the cells were kept under anaerobic conditions. These cells were also shown to possess high rates of aerobic glycolysis. Groth *et al.* (95) have shown that under conditions set up for anaerobic glycolysis, homogenates of Flexner-Jobling carcinoma convert pyruvate to lactate, and to an equal extent to other products, chiefly propanediol phosphate.

Ghosh & Lardy (96) have found in yeast that the inhibition of the Pasteur effect by tumor extracts results from the presence of elemental sulphur. They suggest that the failure to find activity in normal tissue extracts is due to differences in protein structure which make for greater lability of the sulphur in tumor proteins. Wenner & Weinhouse (97) reported that DPN was less tightly bound by tumor and brain mitochondrial enzymes than by these enzymes in other tissues and suggested that this difference might account for the high rates of glycolysis exhibited by tumor and brain. That there are other differences with respect to glycolysis between these tissues and the other normal tissues examined was indicated by the finding of LePage (98) that glycolyzing homogenates (fortified with DPN) of tumor and brain were not influenced by insulin, whereas insulin increased lactic acid formation at low glucose concentrations in homogenates of other tissues studied.

Riley *et al.* (99) have demonstrated the presence in mouse melanoma tissue of a heat-labile inhibitor of cytochrome oxidase activity, a finding which emphasizes the need for more studies designed to detect enzyme inhibitors in tumors.

The possible importance to the cancer problem of enzymes concerned in methyl group metabolism has received some attention (100). Choline oxidase activity, found earlier to be low or absent in rat liver tumors, has been found to be present in two transplantable mouse liver tumors to the extent of 50 per cent of the activity of normal mouse liver (101). Dimethylthetin-homocysteine transmethylase activity is low in liver tumors from both rats and mice (101). Choline oxidase activity is low (102) and dimethylthetin-transmethylase activity is high (100) in the livers of newborn rats. Reid, Landefeld & Simpson (103) observed that the choline of rat liver tumor contained less C^{14} than that from normal or precancerous liver after the injection of L-histidine-2-C^{14}. All the radioactivity of the choline from the tumor was

in the methyl groups, whereas the choline from liver contained 21 to 39 per cent of the radioactivity in the ethanolamine portion of the molecule. Sprinson & Rittenberg (104) also used histidine and found that 40 per cent of the radioactivity in the choline of normal liver was in the ethanolamine moiety. Reid and associates suggest an impairment in ethanolamine synthesis in the tumor as an explanation for their results. Dent *et al.* (105) had previously found large quantities of ethanolamine in the urine of a patient with primary liver cancer and attributed its appearance in the urine to the presence of the tumor.

Beta-glucuronidase activity has been found to be low in rat liver tumors (106). Cholinesterase activity was high in rat liver tumors and low in mouse liver tumors (101), and tributyrinase activity was low in rat liver tumors and high in mouse liver tumors (101).

Adams (107) has found that the injection of tumor homogenates into rats depresses liver catalase activity to the levels obtained in adrenalectomized-castrated animals. As no further depression is produced in adrenalectomized-castrated animals, he suggests that the depression obtained in intact animals is mediated through interference with hormonal control. Appleman *et al.* (108) found liver catalase activity to be low in protein-deficient rats. In these animals the implantation of Jensen sarcoma produced a markedly smaller depression of liver catalase than in animals on a standard diet. Fukuoaka & Nakahara (109), working with a partially purified preparation from tumors which depressed liver catalase activity in rats, report that the catalase depression could be prevented by feeding liver or $FeCl_3$. Hargreaves & Deutsch (110) have observed an *in vitro* inhibition of catalase activity by tumor extracts which also are reported to inhibit other iron-porphyrin enzymes.

Protein and amino acid metabolism.—The decrease in the amounts of "h" proteins (electrophoretic mobility[3] of -1.0 and less) and elevation in proteins of higher mobility (-5.5 and -7), previously found in extracts of azo-dye induced tumors and other tumors [Sorof & Cohen (111)], were not observed either in regenerating rat liver or in the livers of fasted rats (112). A somewhat similar decrease in slow components and elevation in the faster components was found by Hoffman & Schechtman (113) in extracts of the livers of rats fed 4-dimethylaminoazobenzene. Since like changes were observed in the livers of rats fed aminoazobenzene (which is noncarcinogenic), and in the livers of very young rats, the latter authors question the connection between these changes and the malignant state. Their preparative procedure differed considerably from that of Sorof & Cohen, however, and less complete nucleoprotein removal might account for some of their faster moving material. Furthermore, the resolution in their electrophoretic patterns was not sufficient to separate the "h" components. A relative increase in proteins of higher mobility in azo-dye induced liver tumors has also been

[3] Expressed as $cm.^2 \times volt^{-1} \times sec.^{-1} \times 10^{-5}$.

reported by Eldredge & Luck (114). With respect to the relatively low amounts of the "h" proteins in most of the tumors studied by Sorof & Cohen (111) it is of interest that they found considerable amounts of this fraction to be present in the slow-growing mouse hepatoma 112 B. This tumor metabolizes azo dyes (azo linkage cleavage) as rapidly as normal mouse liver [Kensler *et al.* (101)], whereas azo-dye induced rat liver tumors with low amounts of "h" protein possess only a small fraction of the activity of normal rat liver.

De Lamarande & Cantero (115) have found a decrease in the plasma albumin and an elevation in gamma globulin in rats with azo-dye induced preneoplastic livers. In regenerating liver experiments similar changes in the plasma pattern were observed. Such changes are common in patients with liver disease.

Price *et al.* (116) have noted the presence of inclusion bodies in the liver of rats fed azo dyes, and correlated the number with the level of bound dyes. Inclusion bodies, presumably protein (117), have been noted in regenerating rat liver.

Our present knowledge of nitrogen metabolism in tumor-bearing animals has been summarized by Mider (118). The concept that tumors act as a "nitrogen trap" is strongly reinforced by the observation of LePage *et al.* (119) that the proteins of Flexner-Jobling carcinoma were not available for fuel to the host during starvation, although 31 per cent of the body weight and 39 per cent of the liver protein was lost. In rats, proteinuria is affected by sex hormones, and is usually greatly elevated in males with azo-dye induced liver tumors [Linkswiler, Reynolds & Baumann (120)]. The occurrence of gamma globulin of the same unusually low electrophoretic mobility in the plasma and bones of a patient with multiple myeloma supports the view that myeloma cells are the site of formation of the myeloma protein found in the plasma [Miller *et al.* (121)].

The metabolism of amino acids in normal and tumor tissue continues to be actively studied. The uptake of free amino acids by mouse ascites carcinoma cells has been followed by Christensen and associates. When the mice were fed glycine or L-alanine (122) the carcinoma cells were more active in the accumulation of the free amino acid than the cells of liver or muscle; this is considered to be a significant factor in the growth of the neoplastic cell in a wasting animal. Free ascites cells took up L-amino acids when incubated *in vitro* (123); the D-acids were also concentrated though generally not as much as the L-forms (124). *In vitro*, α,γ-diaminobutyric acid was concentrated by the carcinoma cells so strongly as to displace most of the cellular potassium (125). When a moderate dose was given to mice subcutaneously, however, almost all of it was taken up by the liver; it was only after the liver was saturated (with the displacement of half its cellular potassium) that the amino acid was concentrated by the carcinoma. The tumor cells also took up peptides *in vitro* (126). Suspensions of Ehrlich or Gardner lymphosarcoma ascites cells incorporated much more of the radioactivity of C^{14} glycine into protein than suspensions of normal mouse liver cells [LePage (127)]. The up-

take of histidine and other amino acids by slices of rat sarcoma has been studied by Negelein (128). Wase *et al.* (129) observed a more rapid uptake of S^{35} methionine into livers of rats fed AAF.[3]

The amino-acid incorporating system of isolated cell fractions, and its coupling with oxidative phosphorylation, are being intensively studied [Peterson & Greenberg (130); Kit & Greenberg (131); Siekevitz (132)].

Nucleic acids.—Since recent work on nucleic acids is reviewed elsewhere in this volume, only papers concerned directly with tumor tissue will be discussed here. Both the DNA and PNA[2] of mouse ascites thymoma were maximal during the period of most rapid growth [Levy, Davidson & Schade (133)]. The average DNA per cell was doubled in the GRCH 15 tumor of the fowl [McIndoe & Davidson (134)].

Microphotometric measurements on individual nuclei of mouse sarcoma 180 and lymphosarcoma L1 gave DNA values which clustered in groups corresponding approximately to the polyploid classes of normal liver cells, with an average corresponding to tetraploid nuclei [Carnes, Weissman & Goldberg (135)]. Similar grouping of total nucleic acid values into multiples was found in the nuclei of human squamous cancer cells by Mellors, Keane & Papanicolaou (136). In the Ehrlich ascites tumor the DNA content per nucleus was doubled, whereas in ascites lymphoma it was normal [Leuchtenberger, Klein & Klein (137)]; these results agreed with chromosome counts made on the same two tumors by Hauschka & Levan (138). The PNA per nucleus was greatly increased in both (137).

In a study of the incorporation of glycine-2-C^{14} in the rat [Tyner, Heidelberger & LePage (139)], the specific activity of the purines was higher in Flexner-Jobling carcinoma than in liver, whereas the specific activity of the proteins was higher in the liver. In tissue slice experiments orotic acid was incorporated into rat and human tumors much more rapidly than into rat liver [Weed (140)]; *in vivo*, however, Hurlbert & Potter (141) found that rat liver utilized the orotic acid (injected intraperitoneally) very rapidly and suggested that little was available for incorporation into the tumor. Azo-dye induced rat liver tumors incorporated C^{14}-adenine into both DNA and nuclear PNA to a greater extent than normal liver [Griffin, Davis & Tifft (142)].

The elevations in both DNA[2] and PNA concentration found in the liver and other organs of mice, bearing sarcoma 180 [Lombardo, Travers & Cerecedo (143)], are difficult to interpret, since no cell counts were reported; but the concept of a general effect of tumors on nucleic acid metabolism is reinforced by the reports of increased incorporation of both P^{32} [Payne and associates (144)] and C^{14}-formate and glycine (145) into the DNA of the livers of tumor-bearing mice, and of P^{32} into the liver DNA of tumor-bearing roosters [McIndoe & Davidson (134)].

Less mature (and more rapidly multiplying) human white blood and bone marrow cells contained less of an inhibitor for pancreatic desoxyribonuclease than mature cells [Henstel, Freedman & Ginsburg (146)].

Tissue fractionation.—As the study of cell particulates prepared by differ-

ential centrifugation continues to play an important role in current cancer research, some of the recent contributions to methodology will be discussed. With improvement of fractionation techniques the approaches of the chemist and cytologist tend to converge. It is now generally recognized that homogenization procedures must be worked out under careful microscopic control. The percentage of cells broken should be estimated in quantitative work, since tumor cells are often more resistant to fragmentation than normal cells, and erroneous conclusions may be drawn regarding a deficiency of some substance in tumor cells. In the isolation of nuclei careful counterstaining and examination for cytoplasmic tags is advisable. If cytoplasmic components are desired care must be taken not to break up nuclei, since this leads to contamination of the cytoplasm with nuclear fragments. In many cases [Price & Laird (147)] estimation of the amount of substance per cell is necessary for proper interpretation of results.

The best technique for the isolation of nuclei is the subject of sharp debate. Although advocates of the use of nonaqueous media claim that soluble constituents are lost when nuclei are isolated in aqueous media [Allfrey *et al.* (148)], nuclei which still contain all their DNA can be isolated in calcium-sucrose [Mizen & Petermann (149)]; and nuclei prepared by a similar technique retain 92 per cent of a water-soluble enzyme involved in the synthesis of DPN[3] [Hogeboom & Schneider (150)]. Criteria for evaluating isolated nuclei have been discussed by Dounce (151).

In rat hepatoma Allard and associates (152) counted the mitochondria and reported that their number was normal although their total mass was decreased. The mitochondrion and the cyclophorase system have been discussed by Green (153). The major morphological effect of cyanide, which inhibited respiration, mitosis, and growth in tissue cultures of embryonic chick heart, was on the shape and distribution of the mitochondria [Christiansen, Danes & Leinfelder (154)].

Evidence is accumulating on the heterogeneity of mitochondria. Laird, Nygaard & Ris (155) separated rat liver mitochondria into two subfractions which differed in PNA[3] content and succinoxidase activity. Both fractions were stained by Janus green B but only the more easily sedimented fraction reduced the dye to safranin in the absence of added succinate. Novikoff *et al.* (156) separated three fractions of mitochondria, three of microsomes, and two mixtures of both from rat liver. Minor chemical and enzymatic heterogeneity was found among the mitochondrial fractions and marked heterogeneity among the microsomal fractions.

Heterogeneity has also been shown in ultracentrifugal analyses of the smaller submicroscopic units, which have been called macromolecular particles. At least four distinct boundaries, following the microsomes, are seen in rat liver; the two slowest components are found in higher concentration in azo-dye induced tumors than in normal rat liver [Petermann (157)]. The macromolecular components of mouse spleen have been found in increased amounts in both spontaneous and transplanted leukemic spleen of Ak mice

[Petermann & Hamilton (158)]. The principal component, which has a sedimentation rate of about 60 S (157, 158) probably corresponds to the 20 mμ particles found by electron microscopy in extracts of lymphatic tumors of leukemic Ak mice [Gross, McCarty & Cohen (159)] and human lymphomas [Hoster *et al.* (160)]. The "growth" granules found by electron microscopy of cultured tumor cells are generally much larger, ranging from 25 to 150 mμ [Porter & Kallman (161); Selby & Berger (162)].

The Control of Neoplastic Cells

A tremendous amount of effort has been expended on the empirical search for agents to control or selectively destroy neoplastic cells. In the past few years this effort has been rewarded by the discovery of compounds which have a high degree of effectiveness in selectively destroying certain types of transplanted tumors in animals (163, 164, 165) and limited usefulness in the treatment of human disease [Karnofsky (166)]. The material covered in the present review is arbitrarily divided into the results achieved with chemical agents, hormones, and viruses.

Chemical agents.—In view of the remarkable differences in sensitivity of various types of transplanted animal tumors to the known carcinostatic agents, it is not surprising that efforts are now being directed at utilizing these agents as tools to uncover biochemical differences among tumors.

Chemical agents: purines and pyrimidine analogues.—The studies of Hirschberg *et al.* (167) on the enzymic deamination of 8-azaguanine to 8-azaxanthine have shown that liver and other tissues have an activity high enough to account for its rapid metabolism. As the 8-azaguanine possessed carcinostatic activity for four out of seven of the tumors employed by these investigators, while 8-azaxanthine possesses no carcinostatic activity, it is extremely interesting that all three of the resistant tumors possessed high deaminase activity, whereas three of the four susceptible tumors had low or negligible deaminase activity. Kidder and associates (168) have found that 8-azaguanine is incorporated into the PNA of *Tetrahymena geleii.* Evidence in support of the hypothesis that this compound owes its growth inhibitory properties to this incorporation was provided by the observations that it did not inhibit growth of *T. geleii* when the uracil concentration was suboptimal and that under these conditions no incorporation into nucleic acids was detectable. A large series of purine and pyrimidine derivatives has been examined for tumor inhibitory activity both *in vivo* (169, 170, 171) and in tissue culture (172, 173). Of the newer agents tested the 2,4-diaminopyrimidines show the greatest activity *in vivo.*

Of seventy purine and pyrimidine derivatives tested by Stock and associates (174), only 2,6-diaminopurine exhibited the ability to interfere specifically with the synthesis of the Kappa factor in paramecia although several antibiotics, nitrogen mustard, and x-radiation had been found earlier to possess this activity. Because the inhibition of Kappa factor production represents an action on a cytoplasmic self-duplicating entity, it may be important in

terms of the effect of this purine on certain tumor cells. A further observation of interest is the finding of Skipper & Schnabel (175) that 2,6-diaminopurine prevents the inhibition of growth of *E. coli* produced by urethane.

Chemical agents: folic acid antagonists.—An interesting review of folic acid antagonists, which covers the literature to 1950, has been prepared by Petering (176). Skipper and associates (177) have found that the depression by A-methopterin of the incorporation of formate into nucleic acid purines can be partially prevented by folic acid. Although A-methopterin inhibits the incorporation of formate into the visceral nucleic acid purines and thymine of the host, it causes a significant increase in formate incorporation into leukemic cells which have become dependent on A-methopterin for optimal growth (178). Goldthwait & Bendich (179) have shown that aminopterin inhibits the incorporation of formate into nucleic acid purines more than it does the incorporation of preformed adenine. These authors observed that the depression of the formate incorporation in different tissues is directly related to the degree of tissue damage caused by the drug.

The development in leukemic cells of resistance to folic acid antagonists, 8-azaguanine, and other agents presents challenging practical and theoretical problems. Law (180, 181) has provided evidence favoring the assumption that mutation and selection constitute the mechanism by which resistant leukemic cells develop. Both Burchenal and associates (182) and Law (183) have shown that leukemic cells which have acquired resistance to folic acid antagonists or 8-azaguanine remain sensitive to the other effective anti-leukemic agents such as 2,4,6-triethylenimino-*s*-triazine and cortisone. Studies by Hutchison *et al.* (184) on cells resistant to A-methopterin have been extended to *S. faecalis*, an organism in which the resistant strain was capable of substituting certain pteroylglutamic acid analogues for pteroylglutamic acid, whereas parent cultures were not able to do so. Broquist *et al.* (185), in a continuation of this study, have shown that the resistant cells are capable of forming 80 times as much citrovorum factor from pteroylglutamic acid in the presence of ascorbate and formate as can the susceptible cultures.

Several colchicine analogues have been found by Leiter *et al.* (186) to possess more favorable ratios of tumor growth inhibition to toxicity than colchicine itself.

Chemical agents: nitrogen mustards.—Recent studies on the effects of irradiation and the so-called "radiomimetic" drugs emphasize differences in the actions of these agents. The free radicals formed on irradiation in aqueous solutions containing oxygen may be HO_2 [Conway & Butler (187)]. The radicals attack the base or sugar of DNA[2] or both, producing unstable phosphate esters which undergo hydrolysis to inorganic phosphate [Weiss (188)]. This leads to a slow degradation which accounts for the continued loss of viscosity after the cessation of irradiation. The reaction of DNA with bis (β-chloroethyl) methylamine, (HN_2)[2], on the other hand, apparently results in an alkylation of the phosphate groups which leads to a change in the shape of

the DNA molecule rather than a depolymerization [Alexander (189)]. The fact that DNA goes into solution when isolated nucleoprotein fibers are irradiated, but not when they are treated with HN_2 [Rozendaal, Bellamy & Baldwin (190)], supports this hypothesis; but Press & Butler (191) find that HN_2 causes a decrease in the primary amino nitrogen of DNA and in nitrogen precipitable as silver purines after acid hydrolysis.

Koller & Casarini (192) compared the cytological effects of radiation and HN_2 on Walker carcinoma 256 in the rat. The time-courses of the two effects were very different, suggesting that cells at the end of the resting stage were most sensitive to irradiation and least senstive to HN_2. This indicates that HN_2 and radiation act by fundamentally different basic reactions.

Goldthwait (193) has found that HN_2 decreases the incorporation of formate into the DNA of the intestine to the same extent as the incorporation of preformed adenine (in contrast to the differential effect obtained with aminopterin).

Hormones.—Furth and associates (194, 195) have found that the tumor-like growths induced in the pituitary of rats by I^{131} administration are readily transplantable into similarly treated hosts, whereas only one of five strains of tumors proved transplantable into normal hosts. Thus, four of these tumors grew only in the absence of functional thyroid and could be caused to atrophy by thyroid hormone administration. A somewhat similar situation has been shown by Purves, Griesbach & Kennedy (196) to exist in the case of goitrogen-induced thyroid tumors which grew only in thyroxin-deficient animals. The growth of the dependent thyroid tumors could be manipulated by thyrotrophin level changes. Those tumors which became more malignant (independent of thyrotrophin) became morphologically different. It was emphasized that this was not a gradual but a sudden change.

Smith *et al.* (197) have found that growth hormone increased both body weight and tumor growth when administered to C_3H mice bearing a transplantable mammary adenocarcinoma. Millar & Noble (198) reported that a benign fibroadenoma in rats was stimulated by crude anterior pituitary preparations, pregnancy, or small doses of estrogens but inhibited by ovariectomy and large doses of diethylstilbestrol. It was suggested that the stilbesterol effect was mediated through an action on the pituitary. Talalay, Takano & Huggins (199) reported that growth of the Walker carcinoma is inhibited by adrenalectomy, hypophysectomy, and cortisone but not by gonadectomy or pregnancy. Korteweg & Thomas (70) had found a slight inhibiting effect of hypophysectomy on the growth of transplanted mammary carcinomas. Bilateral adrenalectomy has been performed in man in an attempt to arrest tumor growth but has been unsuccessful (200, 201).

Cortisone inhibits the growth of a number of transplanted tumors (202, 203, 204) but has produced complete regression in none. Two recent reports indicate that cortisone has caused the appearance of metastases, although the growth of the primary transplant was inhibited (205, 206). It was suggested that this effect of cortisone is due to a reduction in the

"natural defense mechanisms" of the host. Malmgren *et al.* (207) have found that several carcinostatic agents and several carcinogens, but not their inactive analogues, depressed antibody response in mice.

Viruses.—In conjunction with the hypothesis that cancer is a disease of viral origin, great interest attaches to recent observations on the inhibition of virus multiplication by chemical agents which also inhibit neoplastic growth. Thus 2,6-diaminopurine inhibits Russian encephalitis (208), mouse encephalomyelitis (209), vaccinia (210), poliomyelitis (211), and psittacosis (212) virsuses. 8-Azaguanine inhibits psittacosis (212), and the plant viruses, lucerne mosaic (213) and *Aureogenus magnivena* Black (214). Other nucleic acid derivatives and analogues inhibit mouse encephalomyelitis (209), poliomyelitis (211), and *A. magnivena* (214) viruses, and T_2 bacteriophage (215).

Viruses also have the ability to destroy tumors (216 to 221). The subject has been reviewed by Moore (216). The oncolytic effect is dependent on the presence of active virus; it does not take place in immunized animals, or with inactivated virus. Ability to attack a susceptible tumor may be increased or decreased by continuous passage in tissue culture. The difficulties which must be overcome if viruses are ever to be useful oncolytic agents have been discussed by Southam & Moore (222). The mechanism of tumor destruction by viruses is unknown. Since most, if not all, of the effective viruses are neurotropic, it seems possible that there is some common metabolic product of both tumor and brain which these viruses need for their support, so that, as a result of the competition for it, the host cell is destroyed (216). The association between oncolytic activity and neurotropism in viruses is of especial interest in view of the reported enzymic similarity in tumor and brain discussed earlier (97, 98).

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THE BIOCHEMISTRY OF THE TEETH[1]

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Since publication of the last of these reports in 1946 (1) a very great amount of biochemical work has been carried out on the teeth. Due to lack of space only a small part can be reviewed here. The most significant and promising advances have come from the more detailed information which has been obtained on tooth structure and reactions. The results of more refined methods have opened new paths for understanding the many controversial problems which still exist. Biochemical studies up to 1949 have been summarized in a book by Leicester (2).

Composition and Development of the Teeth

Organic compounds.—Much attention has recently been directed to determining the exact nature of the organic constituents of teeth. Although these are present in relatively small amount, especially in enamel, it is now realized that they play an important part in tooth formation, and probably also in dental caries.

Anderson (3) has determined the total nitrogen content of adult enamel as 0.083 ± 0.021 per cent and indicated (4) that the compounds in which it is contained are acid soluble. Losee & Hess (5) found a total protein content of 0.49 per cent in enamel from crowns with deep pits and grooves, and 0.30 per cent when the pits and grooves were few in number. Pincus (6) showed that groove protein differed from the protein of enamel cuticle and might be developmental material trapped in the groove. Hutton & Nuckolls (7) showed that the surface protein from unerupted third molars stained with ninhydrin reagent, while the protein of the matrix did not. They interpreted this to mean that polypeptides or free amino acids occur in the protein material on the tooth surface. This tends to concentrate in potentially carious areas.

It is generally recognized that the protein of the enamel matrix is a type of keratin relatively low in sulfur. Block, Horwitt & Bolling (8) characterized it as a eukeratin on the basis of its distribution of amino acids, and Losee, Neidig & Hess (9) confirmed this, finding a ratio of histidine:arginine:lysine of 1:3:9, which approached the value of 1:4:12 suggested by Block *et al.* for eukeratins. Bennejeant (10, 11), however, believed that the low sulfur content required that enamel protein be classed as a pseudokeratin. At any rate, paper chromatography has shown the presence in this protein of strong spots for threonine, alanine, serine, leucine-isoleucine, aspartic acid, and proline; medium spots for phenylalanine, valine, glycine, and glutamic acid; and weak spots for arginine, lysine, tyrosine, cystine, and cysteic acid;

[1] The survey of the literature pertaining to this review was concluded in October, 1952.

Histidine, methionine, and hydroxyproline were absent or present in very small amounts (8). Pincus (6) found tyrosine in cuticle and groove protein and also in enamel protein (12). Losee & Hess (5) showed the presence in total enamel protein of 0.60 per cent cystine, 1.01 per cent methionine, and about 5 per cent phenylalanine. It is apparent that the variations reported may be due to different composition of matrix, groove, and cuticle proteins. Further work will be required to clear up this point.

Histochemical studies by Wislocki, Singer & Waldo (13) and Wislocki & Sognnaes (14, 15) have shown that both embryonic and adult enamel matrix contain an acid mucopolysaccharide which appears after the formation of keratin, the first organic compound to appear in the developing tooth (15). Pincus (12) also identified a mucoprotein in enamel and suggested that it was chondroitin sulfuric acid. Developing enamel contains nucleoproteins and alkaline phosphatase and radioactive cystine is incorporated in it (15). The significant change in character of chondroitin sulfuric acid at the zone of calcification, demonstrated by Rubin & Howard (16) for bone, and the role of this substance in binding sodium and calcium suggested by Neuman, Di Stefano & Mulryan (17) indicate that it probably plays an important part in the development of the teeth also.

Marsland (18) showed that the final maturation of enamel to the hard, acid-soluble state begins only when matrix production is complete. He believes that the ameloblasts then change to absorptive organs and obtain the necessary calcium from the pulp through the dentinal tubules. The evidence of Kalnins (19) that rodents with vitamin deficiencies which caused detachment of areas of growing enamel could show normal calcification of such detached areas seems to contradict the idea of Marsland. Further evidence in this direction comes from the experiments of Fleming (20) on transplantation of tooth germs and from the studies of Irving (21, 22) who produced shortened ameloblasts which could not produce final calcification of enamel.

Dentin protein is recognized to be collagen. Losee, Leopold & Hess (23) isolated a total of 15.5 per cent by weight of insoluble protein from ground dentin. Most tooth samples contained a rather constant amount of dialyzable nitrogen compounds. The method of treating the sample could alter the ratio of dialyzing to nondialyzing compounds, however. Stack (24) showed that in recently erupted premolars, collagen accounted for 75 ± 2 per cent of the total nitrogen, about 14 per cent of the protein of dentin. Soluble proteins precipitated by tannic acid made up most of the rest.

Atkinson & Matthews (25) failed to find cystine or methionine in dentin but by paper chromatography (26) showed the presence of aspartic acid, glutamic acid, serine, glycine, alanine, threonine, hydroxyproline, histidine, lysine, arginine, proline, valine, tyrosine, leucine, iosleucine, phenylalanine, and tryptophan. Losee, Neidig & Hess (27) found 19 per cent of glycine, 13 per cent of hydroxyproline (the highest content yet found in a protein), medium amounts of lysine, alanine, arginine, serine, threonine, phenylalanine and 1 to 4 per cent tyrosine. They did not find tryptophan. Bennejeant (10)

found 1.49 per cent of tyrosine. The distribution of amino acids conforms to that of collagen and gelatin (27).

Rogers (28) found in dentin 3.5 per cent of a reducing substance containing hexosamine, localized in the Tomes fibers and present to some extent generally in the matrix. This was confirmed by Wislocki & Sognnaes (15), by Pincus (29), and by Hess & Lee (30). The last two papers mentioned identified the compound as chondroitin sulfuric acid, and Hess & Lee found that the hexose was galactosamine.

Zipkin & Piez (31) found that sound dentin contains 888 ± 103 mg. per cent citric acid and sound enamel 98.7 ± 26.3 mg. per cent. The citrate was present as an insoluble complex which contained calcium and phosphate. Täufel & Krusen (32) showed that citrate is a general constituent of calcified tissues.

Pincus (33) found oxidases and dehydrogenases in pulp and dentin. In dentin these probably occur in the tubular fluid.

Inorganic compounds.—As would be expected, teeth contain minute amounts of a large number of substances. Most of these are probably of little significance. Cruickshank (34) believes that zinc, at least, may play a part in calcification and that variations in its content in teeth may be significant as an indication of tuberculous-prone individuals. Rygh (35) noted that zinc retards calcification in bone and teeth. Fields & Charles (36) showed by spectrographic analysis that in filled teeth such filling materials as silver, zinc, tin, and lead migrate into the tooth structure.

Interest has also grown in the sensitivity of the teeth to radioactive trace elements, as distinct from the radioactive isotopes of such elements as phosphorus and calcium which are major building elements of the tooth. De Ment (37) showed that the natural radioactivity of the teeth is mostly due to K^{40}, with an upper limit of concentration of 10^{-4} per cent. Traces of uranium, thorium, radium, and C^{14} are also present. Teeth take up almost no Ga^{72} according to English & Dudley (38), but Wainwright (39) showed that U^{233}, Pu^{239}, and Am^{241} tend to localize in the dental tissues.

The question of the nature of the calcium phosphate and its associated carbonate in enamel and dentin is closely connected with the nature of these substances in bone. It has been established that dentin resembles bone more closely than does enamel, as has been indicated by Dallemagne & Melon (40), Cartier (41), and Hendricks & Hill (42). There is, however, considerable disagreement as to the exact nature of the salts involved. The arguments for the view that dentin contains a hydrated tricalcium phosphate mixed with calcium carbonate, while enamel is a carbonate apatite, have been ably summarized by Armstrong (43). Many crystallographers feel, nevertheless, that the crystal lattice of the substances present must differ from that of an ordinary hydroxyapatite. McConnell (44) sees the difficulty as one of finding a place for water in the lattice with its highly variable composition. Posner & Stephenson (45) visualized an imperfect lattice [as did Hendricks & Hill (42)] on which phosphate was occluded in greater amounts in bone than in enamel. Neuman (46) felt that the crystal surface was the determining fac-

tor, since its great area permitted the adsorption of a large number of ions which could then react with the medium to which the surface was exposed, either by exchange, recrystallization, or lattice growth. In this way the composition of the crystal would represent that of the medium to which it was exposed. The experiments of Sobel & Hanok (47), and their co-workers (48, 49), reviewed by Sobel (50), confirmed the fact that the composition of enamel and dentin reflected changes in the PO_4/CO_2 ratio of serum, induced by dietary changes. Hodge (51) pointed out that, though there was much evidence for the dynamic activity of tooth and bone salts because of the large surface areas, the constancy of the Ca/P and Ca/H ratios indicated some unifying structural principle in these substances. At the present time no final solution can be offered as to the nature of enamel and dentin salts, but the idea of an apatite lattice with variable substituents and a large surface on which physicochemical and chemical reactions can occur seems to offer the most satisfactory explanation of the observed facts.

Properties and Reactions of Teeth

Solubility.—Zipkin (52) and Sreebny & Nikiforuk (53) showed that chelating agents could decalcify teeth even in alkaline solutions. This avoided formation of gas bubbles which might disrupt fine histological details.

The effectiveness of fluoride in enamel in reducing its acid solubility is well established. Palmer, Overstreet & Sachs (54) and other workers believe that acid fluoride solutions applied topically are more effective for this purpose, but Muhler & Day (55) have shown that this is not the case. In the more alkaline solutions, reprecipitation of calcium fluoride reduces the concentration of calcium found on analysis of the solution.

In general, according to Suess & Fosdick (56), the solubility curves of fluoridized enamel are similar in shape to normal ones but about 0.5 pH units lower. Fluoride treatment renders enamel harder according to Hord & Ellis (57), but Swartz & Phillips (58) showed that in normal teeth there is no relation between hardness and solubility. The effect of fluorides evidently results from a reaction with the enamel. Such a reaction has been demonstrated by the use of electron diffraction by Scott, Picard & Wyckoff (59) who showed the formation of calcium fluoride crystals on enamel surfaces after their immersion in sodium fluoride solutions. Long washing with water removed these crystals. Syrrist (60) did not detect calcium fluoride but found evidence of formation of a fluoroapatite on the surface. Clinically, Finn & De Marco (61) found that the teeth of children who had been drinking fluoridated water showed reduced acid solubility.

Studies by Muhler and co-workers (55, 62) showed that stannous fluoride is more effective than sodium fluoride in reducing solubility. Manly & Bibby (63) tested 147 compounds and found that lead fluoborate was most active in lowering acid solubility. Compounds from Groups III and IV of the periodic table tended to be most effective.

The solvent action of fruit juices and soft drinks on enamel was confirmed by McCay & Will (64). The reported greater dissolving action of such fluids

in the presence of sugars does not exist, and Muller & Gortner (65) showed that only the titratable acidity of the solutions is important. This is determined by the buffering power of saliva and the properties of individual acids. Muller and co-workers (66) showed that the higher erosive action of soft drinks on the lower molars of rats was due to mechanical factors, since rats trained to drink on their backs showed no such erosion.

Permeability.—Atkinson (67) showed that enamel (not dentin) can act as a semipermeable membrane, resisting the passage of large molecules but not of small ions. Sodium chloride took 10 to 30 days to penetrate through enamel. Using cataphoretic methods, Atkinson (68) showed that different areas in a tooth had different degrees of permeability. Penetration was by way of the prism sheaths and decreased with age. Osmotic effects in teeth could therefore be important. Berggren & Hedström (69) showed that addition of glucose or fructose to a solution of tetanus toxin allowed the toxin to penetrate enamel. This effect was not found with organic dyes. Topical fluoride treatment did not change the penetration (70). A similar failure of fluoride to inhibit penetration of substances was observed by Armstrong & Singer (71). Wachtl & Fosdick (72) showed that human enamel was permeable to small molecules or ions, and Wainwright & Lemoine (73) found that radioactive urea penetrated diffusely into enamel in ten minutes. Later Wainwright & Belgorod (74) found that labelled nicotinamide, urea, thiourea and acetamide penetrated 3.5 mm. of dentin to the dentinoenamel junction in 20 minutes. Bartelstone (75, 76) has shown that I^{131} can penetrate enamel, dentin, and cementum diffusely, particularly in abraded or poorly calcified areas. Sognnaes and co-workers (77) confirmed this and showed that H^3 also penetrates from the exterior surface of monkey molars. Amler (78, 79) noted that most medicaments employed in dentistry increased the permeability of dentin to P^{32}. Fluorides at first increased the permeability, but later lowered it.

Radioactive calcium and phosphorus have been studied extensively. Sognnaes & Shaw (80) confirmed that P^{32} could enter the tooth either from saliva or pulp. Ca^{45} is believed by Underwood & Hodge (81) to be taken up by enamel or dentin by an exchange reaction. Singer & Armstrong (82) and Jarabak (83) showed that uptake can occur either from saliva or pulp. Thus, calcium and phosphorus probably behave in the same manner. C^{14} was shown by Armstrong, Schubert, & Lindenbaum (84) to deposit in actively growing portions of bone and teeth.

Dental Caries

As usual, the quantity of literature on caries has been enormous, and only a small part can be reviewed here. It has long been assumed that caries is due to local rather than to systemic factors, but the first direct evidence for this comes from the work of Kite, Shaw & Sognnaes (85). They showed that a diet known to produce caries when fed normally to rats failed to do so when fed by stomach tube.

The importance of carbohydrate fermentation in inducing caries is so

well known that it is not necessary to review this extensively. Hence some of the newer theories of caries etiology will be discussed, since caries is undoubtedly a complex process, and these may well be a part of it.

Forscher & Fosdick (86) demonstrated that the presence of sugar in acid solutions increased the rate of penetration of the hydrogen ion and of lactic acid. This recalls the effect of sugar on the penetration of toxins.

A number of claims have been made in the past that raw sugars are less harmful than refined sugars in causing caries, presumably because of some protective factor which is removed in refining. Shaw (87) and Constant, Phillips, & Elvehjem (88) have now shown that there is no difference in caries production by diets with raw or refined sugars as the carbohydrate component. Dreizen & Spies (89) showed that habitual sugar-cane chewers have a high rate of caries.

Sognnaes (90) found that a high sugar diet in the mother appears to predispose the offspring to caries when the diet is continued in their lifetime. He suggested that some change in the developing tooth is produced by such a diet. Hartles (91) actually observed that a diet containing 67 per cent sucrose increased the absolute amount of calcium and phosphate and lowered the Ca/P ratio in both enamel and dentin, though bone was not thus affected (92). These findings make it necessary to reconsider much experimental material which has been reported in the past.

Hardwick (93, 94) and Hardwick & Manly (95) showed that weak acids had a preferential dissolving effect on carbonate ions in the enamel lattice. A porous lattice could be formed in this way which might adsorb organic matter from saliva and thus slow down further acid attack. This is consistent with the observation of von Fellenberg & Schmid (96) that enamel and dentin of caries-resistant teeth are richer in organic matter than those of carious teeth.

The acids produced in carbohydrate fermentation may not be the only acids which dissolve tooth structure. Atkinson & Matthews (25, 26) noted that carious dentin showed the presence of free aspartic and glutamic acids, while these were not found in sound dentin. They suggested that these amino acids were produced by hydrolysis of dentinal protein and exerted a solvent action on the tooth. Pincus (97, 98) found free sulfate in carious material and suggested that certain bacteria secrete a sulfatase which liberates sulfuric acid from chondroitin sulfuric acid to attack the teeth. Whether acids from such sources can be formed in sufficient amounts to account for much tooth destruction has not been determined.

The theory that proteolytic bacteria destroy enamel matrix as an important step in caries has been supported by histological evidence of Frisbie & Nuckolls (99) and by histochemical studies of Sognnaes & Wislocki (100), who noted that in the initial stages of caries, in areas in which organic substances were exposed by acid etching or poor mineralization, there was a loss of mucopolysaccharide, apparently because of a depolymerizing enzyme. This was followed by penetration of microorganisms between the enamel prisms with destruction of keratin and penetration of dentin.

The brown color, apparently a melanin, which often develops in caries has been supposed to be caused by oxidation of tyrosine by proteolytic organisms. Bennejeant (11) has recently supported this view. Dreizen *et al.* (101) showed that a similar pigment could be produced by exposing tooth proteins to methyl glyoxal or acetol, and Dreizen & Spies (102, 103) later showed that teeth exposed to oral lactobacilli also developed such a pigment. Although the exact nature of this reaction remains to be established, it is clear that it is unnecessary to assume proteolytic organisms as part of the process.

Numerous methods for the control of caries have recently been proposed, but only two have shown any clinical success. The use of ammoniated dentifrices has been less successful than was at first hoped, since the results of a careful study by Kerr & Kesel (104) showed that only about a 10 per cent reduction in caries could be attributed to such a dentifrice.

Fluorides remain the most effective and the most carefully studied agent for caries reduction. They can be applied by topical methods with a reduction of about 40 per cent in caries, as shown by Knutson & Scholz (105), or by drinking water containing 1 p.p.m. fluoride during the period of tooth formation. No mottling is produced under these conditions, and numerous studies, most recently by Ast, Finn & Chase (106), Russell & Elvove (107), and Hill, Blayney & Wolf (108), have shown that this method is more effective than topical application.

The effect of stannous fluoride in decreasing acid solubility of enamel led to its study in topical application, and Muhler & Day (109) showed that it was more effective in rats than sodium fluoride. This indicates that at least part of the action of fluorides is probably because of their effect on acid solubility. The other possible mechanism of their action is to decrease the carbohydrate fermenting power of lactobacilli. That this mechanism is active is indicated by the finding of Finn & Ast (110) that in areas in which fluoride is present in the drinking water, the lactobacillus count is low in children's mouths. A comparable reduction after topical application of fluorides was not found by Kitchin *et al.* (111) or Rickles & Becks (112). Clapper (113) demonstrated that lactobacilli lose their power to produce acid when exposed to fluoride, though they may continue to survive, and Gurney & Rapp (114) showed that the solubility of fluoride from fluoridated enamel was sufficient to establish an appreciable concentration of fluoride ion. Thus the production of sufficient fluoride ion at the immediate site of a caries lesion to inhibit further acid production at that point remains a possibility.

The fluoridation of public water supplies as a measure of caries prevention has raised questions of possible toxic effects if fluorides are retained to too great an extent in the body. Such effects have not been observed clinically, and the explanation is to be found in the studies on fluoride exchange by Largent & Heyroth (115), Jackson *et al.* (116), and Zipkin & McClure (117). These show that fluoride deposits most actively in growing bones and teeth and is taken up more slowly by older organisms. At a constant fluoride in-

take the level of retained fluoride becomes constant. At higher intakes, the proportion retained is less. There is thus little danger that toxic amounts of fluoride would be built up in the body by individuals who consume fluoridated water.

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IMMUNOPOLYSACCHARIDES[1]

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The first composite account of the chemistry of immunopolysaccharides was presented in this review by Haworth & Stacey (1) five years ago. The most outstanding progress was made at that time in the field of bacterial polysaccharides, especially the pneumococcal polysaccharides. In recent years, the immunopolysaccharides in the erythrocytes, and particularly in the different tissue juices, have been studied with at least as great intensity as the bacterial polysaccharides, and considerable progress has been made in the clarification of the structure of blood group substances. The realisation that erythrocytes are able to adsorb and elute several viruses furthered the intensive study of the substrate of the virus-enzyme. This investigation aroused a general interest, since the principles involved in the binding of viruses to mucopolysaccharide or mucoprotein substrates may throw light upon the virus-host cell relationship.

A part of the newer research on immunopolysaccharides has already been surveyed in this journal in the reviews of Grabar (2) and of Mayer (3) within the more extensive field of immunochemistry. On the chemical nature of blood group substances, there are competent reviews by Morgan (4), Bray & Stacey (5), and Kabat (6). A detailed survey of earlier work on virus hemagglutination and virus substrate was made by Hallauer (7). The chemical and immunological aspect of the bacterial polysaccharides was treated recently by Burger (8). The aim of the present review is to provide a short survey of immunopolysaccharide research of recent years, with no attempt to achieve completeness but rather to stress the most important results from the point of view on the one hand of the biochemical, and on the other hand of the biological aspect.

Biologically important observations about immunopolysaccharides often precede adequate definition of the nature of these substances at their first isolation. The application of paper chromatography for the demonstration of individual carbohydrates by Partridge & Westall (9) represents an important advance in defining newly discovered immunopolysaccharides, since this method of identification of hydrolytic products of mucopolysaccharides is very easy to use.

Blood Group Substances

The number of blood group systems demonstrated during the course of recent work on man has been increased to at least eight. Four of them

[1] The survey of the literature pertaining to this review was concluded in August, 1952.

should be mentioned here: the ABO, MN, Rhesus, and Lewis systems. The presence of the type specific substances of the different systems can be shown together with at least one species-specific substance in the red cell stroma by means of the hemagglutination inhibition reaction. Wiener & Wexler (10) assume that the blood group substances are presumably integral parts of the "envelope." According to Morgan (11), it seems quite probable that the mucoid molecules of the ABO group substances are responsible in part for the reversible extensibility of the erythrocyte "membrane." It would perhaps be more cautious to speak of stroma instead of envelope or membrane, since not all of the specific substances incorporated in large protein-lipid-carbohydrate complexes of the stroma must necessarily occupy a surface position. According to Howe (12), the removal of ether-soluble lipids from lyophilized stroma enhances the potency of the stroma in the inhibition of virus and specific blood group agglutination. Disintegration of the large molecules, as well as accessibility of the specific substances, might explain this phenomenon.

The inactivation of blood group receptors belonging to different systems has been studied by Morgan & Watkins (13) by treatment of human erythrocytes with periodate ion. The erythrocytes treated for 15 min. with 0.001 *M* periodate could no longer be agglutinated with M, N and Rhesus sera, whereas the blood group substances belonging to the ABO and Lewis systems could only be inactivated by using considerably higher concentrations of periodate. Although carbohydrates are more easily attacked than proteins, with periodate, these observations merely demonstrate a difference in the behaviour of the Rhesus and MN substances in contrast to the ABO and Lewis substances, but they do not permit any conclusions regarding their nature.

The mucopolysaccharide nature of the ABO substances has been established beyond doubt; the similar nature of the Lewis substances was recently cleared up, whereas the data regarding the chemical nature of the Rhesus and MN substances are quite insufficient. A lipoprotein fraction of the stroma, exhibiting Rhesus activity and called elinin, was defined by Moskowitz *et al.* (14) essentially by means of its method of isolation. The findings of Carter, who isolated a waxy material with Rhesus activity, could not be repeated (3). Even the trypsin inactivation of the Rhesus receptor resulted in contradictory observations. Calvin and collaborators observed with their elinin fraction and Howe (12) with other stroma fractions, that the Rhesus receptor was inactivated by heat and proteolytic enzymes, while other investigators (15, 16, 17) found that the MN but not the Rhesus activity of the red cells was destroyed by proteolytic enzymes. The main difficulty in clarifying the chemical nature of Rhesus and of MN substances is that these do not occur in tissue fluids and in secretions, and that they cannot as yet be separated from protein constituents of the stroma which also carry substances with other immunological specificities. According to our present knowledge, those blood group substances which can be reviewed

under the immunopolysaccharides, belong to the ABO and Lewis systems.

ABO substances.—Of the ABO substances, the A substance has been the most extensively studied. Stacey (18) put the A substance into the second group of the mucopolysaccharides in which are included the polysaccharides containing hexosamine but no hexuronic acid. The Type IV and XIV capsular polysaccharides and the somatic polysaccharide (C substance) of the pneumococcus belong to the same group. The A substance consists of a polysaccharide moiety linked possibly by means of glycosidic bonds to an amino acid residue. The methylation studies of Bray, Henry & Stacey (19) identified N-acetyl-D-glucosamine, D-galactose, L-fucose, and D-mannose in the acid hydrolysis product of the carbohydrate portion. The occurrence of D-mannose might have been accidental since this carbohydrate could not be demonstrated in highly purified preparations at a later period (5, 20, 21).

The major part of recent work regarding the nature and the chemical structure of the ABO substances was carried out in two laboratories, by Morgan and his associates at the Lister Institute, and by Kabat and his collaborators at Columbia University. Both schools were aware that adequate amounts of these substances cannot be ensured for chemical studies if the stroma of human erythrocytes is used as their source. The amount of the group substances in the stroma is exceedingly small, and their primary extraction can only be carried out with alcohol owing to their firm binding to lipoproteins. The occurrence of the group substances in tissue fluids and secretions of human beings and of certain animals in large amount and in water-soluble form, ensured an adequate source for chemical studies. Both laboratories prepared A substances in an equally high grade of purity from human as well as from animal sources.

Some of the analytical data are given here for a highly purified preparation of Aminoff, Morgan & Watkins (22) isolated from freeze-dried human ovarial cyst fluid. This preparation was selected for description because, owing to its derivation, it was certainly free of O substance, and also because it contains 18 per cent D-fucose, the highest value for any sample, according to Kabat (6). Furthermore it gives no cross reaction with Type XIV pneumococcus-immune serum. The substance was isolated by the phenol extraction method of Morgan & King, which avoids high temperatures or extreme pH values. After careful fractional precipitation with ethanol, an electrophoretically homogeneous material with high viscosity was obtained. Examination in the Svedberg ultra-centrifuge revealed that the material is polydisperse. The mean molecular weight of the substance calculated from diffusion and sedimentation data was 260,000 with a frictional ratio 3.2 (23). The acid hydrolysis products contained 57 per cent reducing substances calculated as glucose. N-acetylhexosamine (46 per cent), D-galactose (17 per cent), L-fucose (18 per cent), but no mannose could be shown in the preparation. The total N was 5.7 per cent. The α-amino acid N represents about 38 per cent, and the α-amino-nitrogen 91 per cent of the total N. Eleven amino acids were found, of which threonine represents the largest

component. This substance per se is a poor antigen in rabbits, somewhat better in man; its conversion into potent full antigen can be attained by coupling it to proteins (4, 6). Since this purified preparation contained neither P nor S, in contrast with previous preparations isolated from hog stomachs, and was furthermore dextrorotatory like the hog stomach preparations, whereas A substance obtained from human saliva by Kabat *et al.* (24) was levorotatory, the question must be asked whether the A substances isolated from different sources correspond to the same chemical entity. Earlier work on A substances obtained from numerous human and animal sources (erythrocytes, urine, saliva, gastric juice, amniotic fluid etc.) does not give an adequate answer to this question because of incomplete chemical analysis. Buchanan & Rapoport (25, 26, 27) discovered recently a new and apparently very potent human source in the meconium. However, their preparation does not seem to equal the purity of A substance isolated from human ovarial cyst fluid or from carefully selected individual hog stomach lining (28, 29). Kabat and collaborators (30, 31, 32) showed recently that highly purified A substances isolated from individual hog, horse, and bovine stomach-lining are analytically essentially similar except for a definitely lower L-fucose content in the bovine materials. The horse substances tend to have lower hexosamine and reducing sugar content and higher total and non-hexosamine N than human, hog, or cow materials. Serologically the differences were more accentuated. The human and hog materials were immunochemically closely related; the A substance from hog stomach was, however, found to be a better antigen in human beings of blood groups O and B, than was A substance prepared in a similar manner from human saliva. The horse A preparations cross-reacted to a lesser extent with antisera than did either hog or human A substances. The greatest difference was shown by the bovine substances, which were species-specific as well as blood group specific, and these two specificities were probably linked together on the same molecule.

The main proof for a high grade of purity and for the immunochemical entity of the human and hog A substances was given by Kabat, Baer & Knaub (33) inasmuch as in the anti-A human antibody excess zone, essentially the total amount of both A substances was precipitated. Forty-six per cent of the A substance could be accounted for in the precipitate on the basis of glucosamine and fucose determinations, and it is reasonable to assume that both the galactose and the acetyl were also precipitated by anti-A. Pardee & Blaker (34) demonstrated furthermore that the molecular weight, shape, and size of the gastric mucin A substance corresponds to that found by Kekwick (23) in the human ovarian cyst fluid.

The pure grade of physically and chemically carefully analysed A substances made it possible to begin structural investigations. Bray, Henry & Stacey (19) demonstrated, already in the first methylation studies, that both fucose and N-acetylglucosamine are present as non-reducing end groups in the alkali-stable residues. Oxidation of the A substance with periodate led

Aminoff & Morgan (21, 35) to assume that the smallest unit of the polysaccharide moiety with a reducing end group and a non-reducing terminal residue containing three different forms of fucose is about 3880. This unit, containing four of each of fucose, galactose, N-acetylglucosamine, and N-acetylchondrosamine residues, apparently represents an average and not necessarily a repeating structural unit. A number of such units together with amino acid complexes build up the molecule of the A substance.

An important, and in other chemical investigations unusual, method used in the attempts to clear up the structure of the A substance consisted in the study of its cross reactions with other mucopolysaccharides. Earlier studies had shown that pneumococcus Type XIV antiserum obtained in horses cross reacted to a certain extent with the ABO substances of human erythrocytes, with the Forssman antigen of sheep erythrocytes, and with several other mucopolysaccharides of bacterial and plant origin. It should be noted that the Type XIV pneumococcus polysaccharide contains D-galactose and D-glucosamine in the ratio 1:3, but it does not contain fucose and bound amino acid residues; the polysaccharide moiety of the mucolipid Forssman antigen contains D-glucosamine and hexose. Kabat *et al.* (36) discovered that mild hydrolysis of the ABO substances at pH 1.5 to 1.8 resulted in destruction of blood group activity and a marked increase in cross reactivity with Type XIV antiserum. The dialysate of the hydrolyzed A substance contained about 80 per cent of the fucose initially present, as well as partially depolymerized glucosamine-galactose residues. The non-dialyzable portion exhibited the increased cross reactivity. It was analytically similar to the dialysate except for a much lower fucose content and a greater positive optical rotation. They concluded that both A and O substances are composed of long chains of N-acetyl-glucosamine and galactose residues with the fucose residues projecting outward as end groups at various undetermined points along the chain. Aminoff, Morgan & Watkins (37) hydrolyzing A substance with *N*-acetic acid, came to similar conclusions with regard to the analytical data. They showed furthermore that partially hydrolyzed A substance, by losing its specific A character, reacts more intensively with the Forssman component of sheep erythrocytes, since there is a decided increase in its power to prevent the hemolysis of sheep red cells by rabbit anti-A serum. Further work of Kabat and collaborators (30, 31, 32, 38) demonstrated an inverse correlation between fucose content of hog A and O substances and their ability to cross-react with Type XIV antipneumococcus serum. No similar correlation was found with the human blood group substances, except that the A substance of Aminoff, Morgan & Watkins (22) with the highest fucose content did not cross-react at all with XIV antiserum unless subjected to mild hydrolysis. Mild acid hydrolysis also increased the cross reactivity of horse and bovine substances. A quantitative relationship could not be observed since partially hydrolyzed hog substances cross-reacted better than unhydrolyzed bovine substances, even when the fucose content of the latter was lower. Beiser & Kabat (32) are

therefore of the opinion that some hydrolytic change other than the removal of fucose may also be responsible for the enhancement of cross reactions. The conclusion can be drawn from this interesting immunochemical work that there is a fundamental polysaccharide pattern consisting of N-acetyl-glucosamine and galactose units common to blood group substances and to several other mucopolysaccharides. The removal of the fucose end groups of the blood group substances explains at least partially their increased cross reactivity with XIV antiserum. There is no evidence that the fucose plays a part in the determination of group specificity. The basic structure of the polysaccharide moiety of the substance has been fairly well clarified; but the available experimental data give hardly any information about the nature of the group and still less about the species specificity. It can only be hoped that further studies on the amino acid complex of the blood group substances may throw some light on the chemical basis of the group specificity.

No essential analytical difference can as yet be demonstrated between the purified A and B or O substances. Although the purified ABO substances show a certain degree of serological crossreactivity, according to Morgan (39) this is less than 1 per cent as judged by their agglutination-inhibition end points. The specificity of the serological reactions goes even further, and it is possible to distinguish a spectrum of A and B substances (10). Morgan and his collaborators (40, 41) were able to show, by using human and a series of animal anti-O sera, that such a spectrum exists also in the O substances. Those which reacted differently with a human anti-O isoagglutinine were termed H substances, indicating a difference from the O substance of human erythrocytes and their derivation from different animals as well as from tissue fluids by human secretors. Even with respect to the number and quantity of the amino acids contained in the A and H substances by means of sensitive chromatographic methods, gross differences could not be demonstrated. The only essential difference which can as yet be shown between A and O or H substances, apart from their serological reactions, is in susceptibility towards the enzymes of *Clostridium welchii* culture filtrate. Stack & Morgan (42, 43) purified the enzyme-containing culture filtrate to a degree such that 60 per cent of the original activity associated with 0.2 per cent of the crude filtrate nitrogen was obtained. Their preparation contained a depolymerase acting on blood group substances. The enzymic complex could be differentiated by heating it for 10 minutes at 55°C., whereby depolymerizing activity for the A and B substrate was denatured, leaving other groups unaffected and still able to bring about the inactivation of the O or H serological character.

Lewis blood group substances.—The Le^a substance, discovered by Mourant in 1946, is completely independent of the ABO system with regard to its distribution and inheritance, and can, therefore, be included for chemical analysis, since it occurs plentifully in water-soluble form in the secretors, including the saliva and the ovarial cysts. Grubb (44) found that 41 out of

42 Lewis-negative persons and none out of 20 Lewis-positive persons were secretors for ABO substances. The saliva of Lewis-positive persons inhibits the hemagglutination of the anti-Lewis serum, usually at a high dilution. Morgan (39) and Annison & Morgan (45, 46) isolated the Lewis substance from ovarial cyst fluid with a similar method to that used for the A substance. The only difference found in the chemical analysis of A and Le^a substances was that the latter contained D-glucosamine and D-chondrosamine in a ratio of 3:1 instead of equimolecular proportion. Even the aminoacid chromatograms were indistinguishable, qualitatively, from those given by the group A substance. The levorotatory Le^a mucopolysaccharide is electrophoretically homogeneous, and according to Kekwick (47) its molecular weight and its other physical characteristics are the same as those of the A substance.

The water-soluble blood group substances of the ABO and Lewis systems thus reveal a common or basic molecular framework, but their specific nature is probably determined by minor qualitative or stereochemical modifications of unknown nature.

Heterophile Antigens of the Sheep and Beef Erythrocytes

Complement-fixation reactions, as applied to the Wassermann reaction, are usually carried out with a hemolytic system using sheep erythrocytes and their antibody. The antibody produced in rabbits by means of intravenous injection of sheep erythrocytes reveals a hemolyzing as well as an agglutinating activity. Sheep cells are, however, agglutinated by several other antibodies, e.g. the Forssman antibody or those which occur in the sera of human beings suffering from infectious mononucleosis and serum disease. On the basis of previous serological work, it could be assumed that these antibodies are different. Recent work of Tomcsik & Schwarzweiss (48 to 52) demonstrated the difference by separating some of the antigenic fractions of the red cell stroma. They showed that the immune serum produced by injection of sheep erythrocytes in rabbits contains two antibodies (48), one species-specific antibody reacting with the thermolabile stroma protein, the other the well-known Forssman antibody reacting with the cold alcoholic extract of sheep erythrocyte stroma as well as with the purified Forssman antigen (53). The other stroma antigens reacting with human sera are, however, insoluble in water or in cold organic solvents. For their isolation the stroma of beef red cells was used, being a better source of these heterophilic antigens. With fractional extraction, using first cold organic solvents, later boiling ethanol (first 100 per cent, followed by 80 per cent), two fractions could be obtained, one (100 per cent ethanol) reacting chiefly with serum-sickness serum and the other (80 per cent ethanol) with mononucleosis serum. The fraction reacting with serum-sickness serum contained after acid hydrolysis 8.3 per cent reducing substances calculated as glucose and 4 per cent glucosamine. In the serologically highly reactive "mononucleosis" hapten, 17.4 per cent reducing substances and 8.6 per cent

glucosamine were found. Both substances were resistant to the action of trypsin and pepsin. The small yield of the substances prevented a further purification and a more thorough analysis, but it seems probable that these substances, bound more firmly to the stroma proteins than the ABO substances, belong also to the class of mucopolysaccharides. Apart from the serological interest in separating non group-specific substances from the stroma, these findings may give encouragement to the isolation of the still less known Rhesus and MN blood group substances.

Virus Substrates

Earlier work on the Hirst test led to the following conclusions: (*a*) certain viruses agglutinate a series of erythrocytes, (*b*) the viruses can be eluted from the erythrocyte surface free from most of the tissue material in which they were originally suspended, (*c*) a change in an unknown stroma constituent can be assumed, since after the elution of virus the erythrocytes are incapable of being agglutinated by further addition of virus. Virus-antibodies inhibit it specifically and, in minute amounts, the hemagglutinating action of the viruses of influenza, mumps, and Newcastle disease. It was shown recently that, apart from specifically reacting-immune sera, the extract of the susceptible erythrocytes and a variety of tissue fluids, secretions, and excretions might inhibit to some degree the virus-hemagglutination. The nonspecific inhibitors may be classified as thermolabile and thermostable. The major part of recent work concerns the thermostable inhibitors. These appear to be mucoproteins viz. mucopolysaccharides, and should therefore be briefly reviewed here.

Francis (54) discovered that the thermostable inhibitor substance, which he found in serum of men and of several animals, could be demonstrated in much smaller amount when the virus was previously heated for 30 min. at 56°C., when it had not lost its hemagglutinating property The heated virus could, however, not be eluted from the surface of the erythrocytes. Similarly heated viruses were used as sensitive indicators during the course of subsequent work for measuring the activity of the purified, thermostable virus-substrate. Another indicator used in the characterization of this substrate is an enzyme of the *Vibrio cholerae* culture filtrate, discovered by Stone (55), which destroys the virus inhibitory activity. This enzyme was used by Isaacs & Bozzo (56) to free the ferret serum of the non-specific inhibitor, which might disturb the specific inhibitory effect of the influenza virus antibody.

Non-specific inhibitory property was found in apple pectin (57, 58). However, this polysaccharide inhibited the virus hemagglutination to a less degree than substances isolated later out of different human and animal sources. Burnet *et al.* (59) demonstrated inhibitory activity in serum mucoid, in glandular mucin, in the pseudomucinous ovarial cyst fluid, to the highest degree in Morgan's O substance, and to a lesser degree in A substance. The receptor-destroying enzyme of *V. cholerae* abolished the virus

inhibitory activity, but did not destroy the specificity of the blood group specific substance. They assumed that those viruses, which are characteristically pathogens of mucus-secreting surfaces or gland, possess an enzyme acting on a mucinous substance.

A more direct approach to isolating the virus substrate from red cell stroma is found in the works of Anderson (60) as well as of deBurgh (61) and their associates. The latter worked chiefly with human red cell stroma. Their active material showed a similarity with ABO blood group substances inasmuch as it could be extracted with chloroform and was converted on further treatment into water-soluble form. The most highly purified fraction of which 0.1 μg inhibited one hemagglutinating dose of the influenza virus, contained 2.6 per cent N and, after acid hydrolysis, 50 per cent reducing substances, but no P. It behaved in the ultracentrifuge as a polydisperse macromolecular substance.

Hirst (62, 63, 64) studied the thermostable inhibitor in plasma. The activity was associated with serum mucoid and was readily destroyed by periodate, trypsin, and influenza virus. Stulberg *et al.* (65) isolated from human plasma, by the perchloric acid filtrate method, an electrophoretically homogeneous mucoprotein which showed almost the same amount of inhibitor activity as the stroma extract of deBurgh (61). According to Hirst (64) the serum mucoid is at best an ill-defined mixture, and it is by no means certain that it contains only mucoid. It should be pointed out that, independently of virus substrate studies, certain advances were made recently in defining the "serum mucoid." Staub & Rimington (66) isolated two apparently homogeneous substances from serum mucoid, by phenol fractionation. One was apparently a glucoprotein and the other had a much lower nitrogen content, and was a mucopolysaccharide. The carbohydrate moieties of these two fractions are distinctly different, both with regard to their glucosamine contents and the nature of their hexoses. Winzler and associates (67 to 70) described also a homogeneous mucoprotein in human plasma which showed a very low isoelectric point, most likely due to its content of sulfuric acid esters in combination with carbohydrate components. Its molecular weight was found to be 44,100.

A highly active substance of egg white inhibiting virus hemagglutination was studied by Beard and associates (71, 72, 73). This substance seems to be identical with the ovomucoid β, also known as ovomucin. Fazekas & Gottschalk (74) found, however, that only 5.1 per cent of their ovomucin preparation could be absorbed on specially prepared virus-coated erythrocytes, corresponding to the active material, whereas the remainder consisted of a noninhibitory mucoprotein and lysozyme. It is especially interesting that the enzyme of influenza virus, according to Gottschalk (75), splits off only 1 per cent of the ovomucoid, and this corresponds to an isoglucosamine amino acid complex.

The most instructive information on the virus substrate was gained by the study of the inhibitory substance isolated by Tamm & Horsfall out of

normal human urine (76, 77). The isolation and purification of this substance is relatively simple and produces a mucoprotein in good yield with a molecular weight of 7×10^6 consisting of thread-like molecules which have axial ratios of approximately 100. 12.6 per cent N, 5.3 per cent P and after acid hydrolysis 33 per cent reducing substances and 9.2 per cent glucosamine were found. The minimal amount of the substance capable of giving a demonstrable reaction with one hemagglutinating unit of virus was as low as 0.0003 μg. After treatment with influenza virus its biological activity was eliminated, but the substance remained homogeneous and its electrophoretic activity was decreased by approximately 20 per cent (78). The macromolecular urinary mucoprotein is antigenic in rabbits, as was shown by Fraser (79) and by Tamm & Horsfall (77). The antibody gave the usual serological reactions with the urinary substrate but did not react with erythrocytes. The inhibitor activity of the urinary mucoprotein was suspended by the addition of antibody; this is no reason to suppose that such antibodies combine with those groupings of the molecule which react with viruses. It is significant that the receptor-destroying cholera enzyme abolishes the inhibitory activity but does not influence the serological reactivity of the substance. This observation indicates that inhibition of virus hemagglutination may be a function of the antigenic macromolecule dependent on molecular groupings which are not antigenic determinants. The findings of Gottschalk (75) make it also probable that the action of enzymes on the substrate does not lead to a drastic change. This explains why, according to Burnet (59), the receptor-destroying enzyme does not alter the O blood group activity by abolishing its inhibitory activity for virus hemagglutination. It is, however, not known what molecular groupings may be similar in the O mucopolysaccharide and in the urinary mucoprotein, to account for the inhibitory action on the virus hemagglutination in both substances.

Another type of important virus substrate was demonstrated by Ginsberg, Goebel & Horsfall (80, 81, 82). Continuing the work of Horsfall & McCarty, they showed that as little as 5 μg of the Friedländer bacillus Type B polysaccharide inhibited the multiplication of mumps virus in the allantoic sac of the chick embryo. The structural configurations of the polysaccharide responsible for specific serological activity are not identical with those which determine the inhibitory effect. The polysaccharide is also effective as inhibitor of the mumps virus hemagglutination, but this property depends probably upon a different molecular configuration, since HIO_4 treatment eliminates only the inhibitory activity for hemagglutination, and alkali treatment only that for virus multiplication. The multiplication of influenza and Newcastle disease viruses is not inhibited by the Friedländer polysaccharide. Ginsberg & Horsfall (83, 84) assume that the two groups of viruses may require different host metabolic systems for multiplication and that the polysaccharide blocks only the mumps virus receptors of susceptible host cells.

Microbial Polysaccharides

Stacey (18) expressed the opinion that a small amount of polypeptide constituent in the pneumococcus polysaccharides may determine their antigenicity. Heidelberger and associates (85) succeeded, by means of mild procedures which hardly changed the original relative viscosity, in obtaining a highly purified pneumococcus Type III polysaccharide with as little as 0.05 per cent of N and 0.01 per cent P. This preparation exhibited the high antigenicity expected in man, thus the possible role of a hypothetical, N-containing prosthetic group in the determination of the antigen action was disproved. Because of cross reactions between the pneumococcus Type III polysaccharide and the polysaccharides produced by *Azotobacter chroococcum* and *Rhizobium radicicolum*, the chemical structures of the latter substances were studied by Lawson & Stacey (86). Similarity was found with *Azotobacter* polysaccharide in the possession of the glucose-glucuronic acid type of aldobiuronic acid residues and in a significant proportion of 1:3 glycosidic linkage. The relationship to the *Rhizobium* polysaccharide lies in the aldobiuronic acid residue.

By immunization of human beings with minute amounts of pneumococcus polysaccharide, the peculiar observation was made that reinjection was not followed by the usual secondary increase in antibody content. As reviewed by Grabar (2), a theory was developed that under certain conditions the type-specific polysaccharide might "paralyze" antibody formation. Heidelberger and associates (87) and Murray *et al.* (88) demonstrated recently that reinjection of the polysaccharide at an appropriate period enhances the antibody content or the protection of experimental animals toward infection, thus there is no need to assume a "paralyzing" effect.

Continuing their pneumococcus enzyme studies, Sickles & Shaw (89) differentiated two strains of *Bacillus palustris*. One of these splits specifically only the Type III, the other both Type III and VIII pneumococcus polysaccharide. Antisera suspending the biological activity were produced against both enzymes, and these also revealed a type specificity. One of their strains produced the polysaccharide-decomposing enzyme also in the absence of substrate (90). Horsfall (91) showed that an enzyme which can be separated from *Streptococcus salivarius* can synthesize an immunologically active levan in the presence of sucrose. This levan is serologically similar to those synthesized by members of the *Bacillus* group, but unrelated to the capsular polysaccharides of the microorganisms which produce them. Pierce & White (92) observed that *Streptococcus pyogenes* produced in liquid media containing galactose only one-third to one-half of the hyaluronic acid produced on media containing glucose.

The group specific *Streptococcus* A substance was isolated earlier with drastic methods of extraction, such as HCl at 100°C. or formamide at 150°C. It was questioned if the relatively small molecular weight, *ca* 8,000, of such a preparation might not be the result of a depolymerization. Schmidt (93) in-

vestigated this problem and showed that this group specific *Streptococcus* polysaccharide can be isolated from ground streptococcal cells with essentially the same molecular weight. 2.6 per cent N, 83 per cent reducing substances after hydrolysis (calculated as glucose), 60.8 per cent rhamnose, and 26.1 percent glucosamine were found in this substance, showing the same composition as the *Streptococcus* A substance isolated by drastic methods. After an intravenous injection with a fluorescent-labelled antibody, the cellular localization of this polysaccharide was limited to the tubular epithelium of the kidney. Bacterial mucopolysaccharides of high molecular weight, on the other hand, were distributed widely and could be demonstrated particularly in the cells of the reticulo-endothelial system.

It had been noted previously that the smooth form, or Phase I, of *Shigella sonnei* readily dissociated in Phase II varieties S and R. The serologically-different Phase I S and Phase II R lipocarbohydrate-protein complexes were isolated by Baker, Goebel and Perlman (94). Goebel and associates (95, 96, 97) showed that the Phase II complex is the specific substrate for the action of certain members of the T series of bacteriophages. The serological specificity of the somatic antigen seems to play a role determining the specificity of the bacteriophage substrate, since *Escherichia coli B* contains a lipocarbohydrate-protein complex serologically similar to that of Phase II substance, and this bacillus is sensitive to the same bacteriophages as Phase II dysentery bacillus. Furthermore the Phase II substance inhibited the action of certain bacteriophages on *E. coli B* (98).

Westphal *et al.* (99) isolated a N-containing polysaccharide of *Salmonella typhi* through phenol extraction. This polysaccharide exhibited an unexpected antigenicity in rabbits. It should, however, be recalled that Morgan & Partridge had already found a similar action in the phenol extract of dysentery bacilli.

Keogh and associates (100, 101) discovered that bacterial polysaccharides obtained from *Pneumococcus, Streptococcus, Neisseria, Haemophilus,* and *Salmonella* groups of bacteria are adsorbed to erythrocytes and cannot be washed away with neutral saline. The coated erythrocytes, showing agglutination or hemolysis after the addition of the homologous polysaccharide-immune sera, were found to be useful reagents in subsequent bacteriological and clinical work. Especially significant was the finding of Middlebrook & Dubos (102) that sheep erythrocytes treated with a polysaccharide fraction of tubercle bacilli reacted with the serum of patients suffering from active tuberculosis. A modification of the test, involving an inhibition reaction, has been developed for the detection of minute amounts of polysaccharide. Pound (103) reports, however, on non-specific reactions, using erythrocytes coated with phenol-extract of tubercle bacilli.

One of the best known bacterial polysaccharides, dextran, produced by *Leuconostoc mesenteroides*, was reviewed by Haworth & Stacey (1). They defined the dextrans as polyglucoses produced from sucrose by a variety of organisms. Hehre & Hamilton (104) showed that *Acetobacter viscosum* and *A. capsulatum* are capable of synthesizing this dextran grown on a dextrin,

but not on other carbohydrate-containing culture media, whereas *Leuconostoc* synthesized dextran only from sucrose. The dextran produced by *Acetobacter* was serologically similar to the *Leuconostoc* polysaccharide and reacted also with the pneumococcus Type II and XX antisera. As was shown by Hehre (105), the Acetobacter-dextran is a high molecular weight polymer of D-glycopyranose units linked principally in α-1,6 positions. The synthesis of this substance from dextrins could also be achieved *in vitro* by the action of a soluble enzyme system, dextran-dextrinase, obtained from *A. capsulatum* cultures.

A cross reaction of *Leuconostoc* dextran with *S. typhi* antiserum had already been observed in an earlier work of Zozaya. Neill & Abrahams (106) confirmed this observation recently, using both unhydrolyzed or partially hydrolyzed so-called "clinical form" of *Leuconostoc* dextran. The clinical use of dextran for blood volume extension as a plasma substitute necessitated the partial breakdown of the high molecular complexes into fragments of about 75,000 molecular weight. For this purpose the enzyme of an *Aspergillus* species isolated from soil was used by Whiteside-Carlson & Carlson (107). Hehre & Sery (108) showed that gram-negative anaerobic bacteria which constitute an appreciable part of normal fecal flora are responsible for the enzymatic breakdown of dextran in the feces. Koepsell & Tsuchiya (109) attempted another method to prepare dextran with molecular weight appropriate for clinical use, by stopping the enzymatic synthesis of the substance at 75,000. Owing to its clinical importance a number of workers studied the reactions produced by injections of dextran into experimental animals and into human beings. The effect of dextran influencing the sedimentation rate of blood cells parallel with the molecular size should be mentioned here (110).

A series of papers have established that *Torula histolytica*, a yeast-like microorganism causing meningo-encephalitis, belongs to the class of capsule-producing microorganisms, in which the capsular polysaccharide is responsible for the type specificity. Difficulties in its serological classification are pointed out in an initial paper by Kligman (111). Recent serological work by Neill *et al.* (112, 113, 114) and particularly by Evans (115) showed three serological types. Chemical analysis of the capsular substance was made by Mager (116), Drouhet *et al.* (117), and Evans and co-workers (118, 119). Xylose, mannose, galactose, and uronic acid were demonstrated by paper chromatography in the hydrolytic products of the capsular polysaccharide.

The virulence-enhancing effect of gastric mucin was reviewed by Olitzki (120). Landy & Batson (121) believe that the active factor corresponds to the blood group A substance, the effect of which is augmented by inorganic salts. Smith (122 to 125), however, concluded that the A substance corresponds only to the viscosity factor and can be replaced by tragacanth. The second factor of the complex activity is an electrophoretically homogeneous peptide-like substance with 5 to 10 per cent carbohydrate content, and the third factor is an unidentified component which can be replaced by charcoal.

Morphological Arrangement of the Immunopolysaccharides in the Bacterial Cell

Fundamental reviews (126, 127) have discussed the difficulties in demonstrating the spatial arrangement of immunopolysaccharides within the bacterial cell. Direct staining of the cellular polysaccharides was attempted recently by Jorpes *et al.* (128) and by Hotchkiss (129) by oxidation with periodic acid and by combination of the resulting aldehydes with fuchsin-sulfurous acid. It was supposed that two adjacent free OH groups were responsible for a positive reaction. Jeanloz (130) could not affirm this assumption. According to him this reaction is unsafe for the identification of polysaccharide structure. Further study of this method with various microorganisms offers promise.

The bulk of information upon the morphological arrangement of polysaccharides was obtained by immunochemical analysis of bacteria in mucoid, smooth, and rough phase. The data concerning the capsule are the most convincing, since the extraction of the water-soluble substance of this surface structure is the easiest, and its identity with capsular material can be checked morphologically by means of the so-called capsule-swelling reaction of Neufeld. The capsules of unstained bacteria, suspended in saline, are not to be seen in the light-microscope, but they show up beautifully and—as is generally believed—in "swollen" forms if antibody, reacting with the capsular material, is added to them. This activity of the immune serum goes parallel with its agglutinating power and can be suspended by addition of the purified capsular substance. Type specific polysaccharides were demonstrated by this means in the capsules of *Pneumoccocus*, *Klebsiella*, *Neisseria*, *Haemophilus*, *Torula histolytica* etc. Polysaccharides without type specificity are found, e.g. in the hyaluronic acid capsule of *Streptococcus*. By using the capsule-swelling and agglutination test, Brooke (131, 132) isolated recently 27 new *Klebsiella* types, increasing the number of capsular types in this group of microorganisms up to 41. Dawson & Zinnemann (133) in England obtained, by the bacteriological analysis of 868 swabs from children of zero to four years, 31 capsulated *H. influenzae* strains, belonging to six serological types, on the basis of their capsular polysaccharide. Ewing *et al.* (134) isolated capsulated forms of *Shigella boydii* 1 and 2 and showed that the latter is related on the basis of the capsule-swelling test to *Klebsiella* Type 21. Most of the "swelling" reactions are carried out with imune sera of rabbits, immunized with killed capsulated microorganisms. Schwab & Shaffer (135) report that such an immune serum can be prepared more easily in chickens.

The nature of the capsule-swelling reaction has not been cleared up adequately. Johnson & Dennison (136) demonstrated by careful measurements an unmistakable increase in volume of pneumococci following the addition of the corresponding antibody; and they suggest that capsular hydration takes place as a result of antigen-antibody combination. Neither Neill and his collaborators (112, 114) with *Torula*, nor Tomcsik (137) with members of

the *Bacillus* group, found a noticeable increase in the size of the capsule after the addition of the immune serum. The reviewer is of the opinion that the essential feature of this reaction is precipitation. This accounts for the visibility of the capsule, whereas the "swelling," due probably to capsular hydration, is a secondary phenomenon, manifesting itself more rapidly in case of certain microorganisms, particularly pneumococci. It would be more correct to speak of specific capsular reaction. The specificity of this reaction is restricted to the pH range 6 to 8. Jacox (138) showed that at pH 4 several proteins are able to induce a similar but nonspecific reaction with pneumococci, but not with *Klebsiella* or with capsulated *E. coli* or *Streptococcus*. Tomcsik & Guex-Holzer (139) observed a similar phenomenon recently with capsulated members of the group Bacillus; the nonspecific protein combination could, however, be dissociated below pH 2 and above pH 4. Non-specific reactions of varying intensity were obtained approximately between pH 2 and 4.

A complex structure of the bacterial capsule was not demonstrated until recently. Wood & Smith (140, 141) showed that pneumococcus Type III is more resistant to surface phagocytosis than any other organism since it possesses a particularly large capsule. They found in this capsule an outer slime layer which stains metachromatically with methylene blue. The metachromatic staining might indicate a different physico-chemical state in the surface slime layer, but no evidence was found for a chemical difference between the surface and deep layer. Williamson & Zinnemann (142) surmised, on the basis of purely serological evidence, two distinct capsular antigens in *H. influenzae*, type e strains. Two chemically distinct substances with different spatial arrangements were discovered in the capsule of *B. megatherium* and in a related bacillus by Tomcsik (143). Tomcsik & Guex-Holzer (144) showed that one of these substances is the polypeptide demonstrated by Bodon & Tomcsik with a similar reaction in the capsule of *B. anthracis*. Unlike the homogenously divided polypeptide, a polysaccharide was also found in this capsule showing a framework with very distinct cross-septa. These cut across the capsule of the bacteria arranged in chains. Recent work (145) has shown that capsular cross-septa are a direct continuation of the cross cell wall. The cross-septa are invisible and the cell wall itself is barely visible in phase-contrast microscope but both show up beautifully after the addition of polysaccharide antibody. The reviewer believes that the cell wall can be demonstrated by means of antibodies with an essentially similar reaction as the bacterial capsule, at least in the case of *B. megatherium* and related microorganisms. The prerequisite for the morphological demonstration of the cell wall by means of antibody is the separation of the cell wall from the cytoplasmic membrane (for instance by lysozyme activity) or partial autolysis of bacteria. Since the polysaccharide obtained by cautious extraction of these bacteria, and freed from polypeptide, inhibited the specific reaction of the capsule as well as that of the cell wall, it is not unlikely that the same polysaccharide which forms the capsular framework participates

in building up the cell wall. It is certainly worth while to apply this new reaction to the clarification of the chemical nature of the cell wall. Thus, Holdsworth (146) disintegrated *Corynebacterium diphtheriae* mechanically until no further material was extracted by 2 per cent sodium acetate solution. The residue was considered to be the cell wall. Its chemical analysis revealed a polysaccharide moiety with a molecular weight of 1200, containing 2 residues of D-galactose, 1 of D-mannose and 3 of D-arabinose and a protein portion. The composition of this protein was characteristic inasmuch as it contained glucosamine and diaminopimelic acid, substances not occurring in the presumed cytoplasmic fraction. The evaluation of these findings is difficult as it is improbable that as much as 45 per cent of the dry weight of the cell would belong to the cell wall. It is even more difficult to evaluate the work of Harris (147) regarding the cellular structure of *Streptococcus*. He disintegrated the cells by sonic vibration. The fraction believed to be the cell wall contained the type-specific M protein. Group-specific carbohydrate, lipoid, and nucleoprotein of the ribose type were shown in the cytoplasmic particles. A supernate fraction contained two components, one of which was part of a dissociable nucleoprotein of the desoxyribose type.

The major part of the previous work regarding the morphological arrangement of the O polysaccharide complexes in the bacterial cell was reviewed by Dubos (126) and in a symposium in London (127). The relation of these complexes to the osmotic barrier of the bacteria is unknown. Lankford and associates (148) made use of the Hotchkiss-McManus periodate-fuchsin stain for polysaccharides by 90 strains representing 8 genera of Enterobacteriaceae. Most of the smooth cultures showed deeply-stained bodies located in one or more, usually in both poles of the cytoplasma, whereas the rough forms could not be stained. It may be hoped that further studies of specific stains might throw additional light on the role of these complexes in cell structure.

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CHEMISTRY OF THE FUNGI[1]

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The biochemistry of the fungi was last considered in the *Annual Review of Biochemistry* by Tatum (1) in 1944. With constantly increasing interest in the subject, an enormous amount of work has been reported in the nine years which have elapsed since that review. An exhaustive treatment of the material available would, therefore, be quite out of the question in the space at our disposal. Rather is it the reviewer's intention to select only those aspects of the subject where, in his opinion, the most interesting and outstanding advances have been made, and to avoid as far as possible the compilation of a mere annotated catalogue of the literature. It is hoped, however, that the conspectus presented will afford an indication of the main trends in the study of fungal biochemistry during the period under consideration, apart from the subject of antibiotics which has already received adequate attention.

Fortunately the many gaps in the present account can be filled in by the information contained in the text book (2) and the supplementary review article (3) by Foster and in the reviews on the particular aspects of the subject such as metabolic products [Raistrick (4)], trace elements [Steinberg (5)], commercial production of fungal acids [Cochrane (6)], acid formation [Walker (7)], biochemistry of *Fusaria* [Nord & Mull (8)], genetics and use of mutants [Chodat (9); Horowitz (10); Mitchell (11); Bonner & Yanofsky (12)], and others mentioned later in this article.

The subject matter will for convenience be arranged in four main divisions which are occasioned largely by the different methods of approach to the study of the fungi: (*a*) Isolation of new metabolic products and correlation with those previously known; (*b*) Study of the mechanism of the metabolic processes by the older (classical) methods; (*c*) Application of genetics and development of the use of fungal mutants; (*d*) Application of isotopes as tracers in the study of fungal metabolism.

NEW METABOLIC PRODUCTS

Aliphatic Series

Ethylene.—It is well established that respiring plant tissue produces ethylene, which in turn promotes the ripening of fruit. Biale & Shepherd (13) and Williamson (14) have shown that *Penicillium digitatum* produces an active emanation when cultured on potato-dextrose agar. Hall (15) on the basis of quantitative determination and by use of the etiolated pea test concluded that the active substance was ethylene. It was produced both

[1] The survey of the literature pertaining to this review was concluded in September, 1952.

by the actively growing cultures of the fungus on dextrose- or arabinose-agar media and by crude enzyme preparations from the mold using a number of substrates which would be found in ripening fruit. Ethylene is also claimed by Nickerson (16) to be a metabolic product of the pathogenic fungus *Blastomyces dermatitidis*. The quantity obtained amounted to 420 mg. from a 3 l. liquid culture in 30 days.

From the fact that the highest yield of ethylene was obtained when the substrate was ethyl alcohol, Hall concludes that the various substrates (carbohydrates, organic acids, alcohols) are eventually degraded to ethanol which is the immediate precursor of ethylene; the final reaction is envisaged as a simple dehydration: $CH_3CH_2OH - H_2O \rightarrow CH_2 = CH_2$, as in the chemical preparation of ethylene. If this is the case however, then with methanol, which is a good source of ethylene, a condensation involving a linkage between the carbon atoms of two single-carbon molecules must occur. It may be of some significance that ethyl acetate is a metabolic product of *P. digitatum* [Birkinshaw *et al.* (17)]. The most likely precursor of ethyl acetate would be acetaldehyde which could give rise to ethanol and acetic acid by a Cannizzaro reaction. It appears that the mechanism of the formation of ethylene would repay further investigation.

Acetylenic compounds.—For some time it has been recognized that acetylenic derivatives occur in the higher plants and several have been isolated and characterized and their structure determined. In 1935, the Russian chemists Wiljams *et al.* (18) isolated from *Lachnophyllum gossypinum* a highly unsaturated crystalline ester to which they assigned the constitution:

$$CH_3\text{—}CH_2\text{—}CH_2\text{—}C{\equiv}C\text{—}C{\equiv}C\text{—}CH{=}CH\text{—}COOCH_3$$

Other similar products from *Matricaria*, *Artemisia*, and *Erigeron*, all genera of Compositae, have since been identified by Sörensen and co-workers (19 to 24). Thus matricaria ester from *M. inodora* has the constitution:

$$CH_3\text{—}CH{=}CH\text{—}C{\equiv}C\text{—}C{\equiv}C\text{—}CH{=}CH\text{—}COOCH_3,$$

whereas another constituent of the same plant known as cumulene hexahydromatricaria ester, an oil, has the allene grouping:

$$CH_3\text{—}CH_2\text{—}CH_2\text{—}CH_2\text{—}CH_2\text{—}CH{=}C{=}C{=}CH\text{—}COOCH_3$$

The oil from the nuts of the boleko tree, *Ongokea Klaineana* contains erythrogenic acid, which was found by Castille (25) to possess two acetylenic linkages and one ethylenic bond in the molecule. A natural acetylenic carbonyl compound and hydrocarbons are also known.

The acetylenic group has now been detected in fugal metabolic products. Several highly unstable antibiotic substances have been isolated from *Basidiomycetes* by Anchel *et al.* (26). Their characteristic ultraviolet absorption spectra have enabled them to be classed as acetylenic compounds. From the absorption data, by comparison with known substances, Sörensen suggests that the products of Anchel (27) possess the following groupings·

nemotin and nemotinic acid, —CH=C—C≡C—C≡C—; compounds isolated from *Clitocybe atreta*, —C≡C—C≡C—C≡C—C=CH— and —C≡C—C≡C—CH=CH—CO_2R; agrocybin, —C≡C—C≡C—C≡C—CO_2R.

Another crystalline antibiotic, mycomycin, obtained from an actinomycete by Johnson & Burdon (28) has now been investigated by Celmer & Solomons (29) and shown to have the constitution 3,5,7,8-tridecatetraene-10,12-diynoic acid:

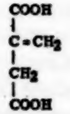
$$HC{\equiv}C{-}C{\equiv}C{-}CH{=}C{=}CH{-}CH{=}CH{-}CH{=}CH{-}CH_2{-}COOH.$$

Mycomycin undergoes an unusual rearrangment in N-KOH at 27° involving allene to acetylene conversion, accompanied by migration of existing acetylenic bonds, to yield isomycomycin with the structure:

$$CH_3{-}C{\equiv}C{-}C{\equiv}C{-}C{\equiv}C{-}CH{=}CH{-}CH{=}CH{-}CH_2{-}COOH.$$

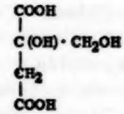
Carotenoids.—The occurrence in *Phycomyces blakesleeanus* of relatively large amounts of β-carotene and traces of α-carotene has been established for some time but closer investigation has shown that other carotenoids also occur in small amount. Schopfer & Grob (30) found that five carotenoids were present in their strain of *Phycomyces*, and Goodwin (31) has examined these minor constitutents more closely. He was able to identify with certainty phytofluene, γ-carotene, ζ-carotene, neurosporene, and lycopene in addition to α- and β-carotene already recorded; phytoene is probably present also, in addition to unidentified substances. Addition of diphenylamine (1/40,000) to the culture solution almost completely inhibited the production of the most unsaturated carotenoids, α-, β-, and γ-carotene and lycopene, whilst stimulating the production of the more saturated compounds, phytofluene, ζ-carotene, neurosporene, and possibly phytoene. From work on tomatoes Porter & Lincoln (32) concluded that lycopene is the parent $C_{40}H_{56}$ carotenoid and that it is synthesised from the saturated compound tetrahydrophytoene via phytoene, phytofluene, ζ-carotene, and neurosporene by the stepwise removal of four hydrogen atoms; α-, β- and γ-carotenes then being produced by isomerisation of lycopene. The inhibition of the formation of the most unsaturated polyenes by *Phycomyces* and the stimulation of the synthesis of the more saturated ones by the action of diphenylamine might be taken to confirm the suggested method of biogenesis, but, as Goodwin points out, it is also capable of another explanation. All the polyenes might be derived by parallel syntheses from a common precursor. Thus blockage of the synthesis of the more unsaturated group would leave more precursor available for the more saturated group of polyenes.

Itatartaric acid.—The survey of strains of *Aspergillus terreus* for the production of itaconic acid by the Northern Regional Research Laboratory, Peoria, Illinois, led to the observation that some of the strains examined formed considerable amounts of saturated acids as well as itaconic acid (I). One of these strains, a mutant obtained by ultraviolet irradiation was found to produce a mixture of acids which were fractionated as methyl



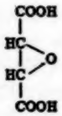
I

Itaconic acid



II

Itatartaric acid

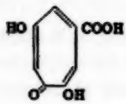


III

Ethylene oxide $\alpha\beta$ dicarboxylic acid

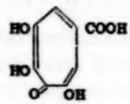
IV

Tropolone



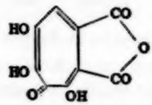
V

Stipitatic acid



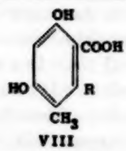
VI

Puberulic acid



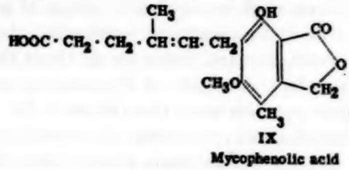
VII

Puberulonic acid



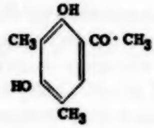
VIII

P. brevi-compactum products



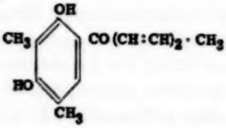
IX

Mycophenolic acid



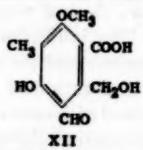
X

Clavatol



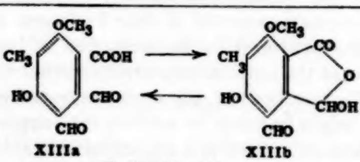
XI

Sorbicillin



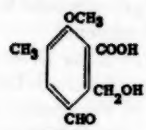
XII

Cyclopolic acid



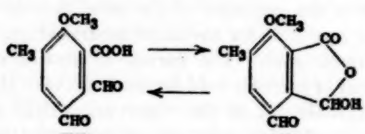
XIIIa XIIIb

Cyclopaldic acid



XIV

Dihydrogladiolic acid



XVa XVb

Gladiolic acid

esters. One fraction, b.p. 129–134°/2 to 3 mm. was shown to be the methyl ester of itatartaric acid (II) [Stodola *et al.* (33)].

Racemic itatartaric acid is known and can be produced synthetically by oxidation of I. It apparently exists only in the form of salts, the γ-lactone form however is a crystalline solid. A higher boiling fraction (b.p. 151–154°/2 to 3 mm.) of the methyl esters obtained from *A. terreus* is presumably the lactone methyl ester of II. The relationship between I and II is the same as that existing between fumaric acid and tartaric acid. The former is a well-known fungal metabolic product but the occurrence of the latter as a product of sugar metabolism by fungi is doubtful. However the dehydrated form, ethylene oxide-$\alpha\beta$-dicarboxylic acid (III) was isolated in crystalline from as a product of *Monilia formosa* and of *Penicillium viniferum* by Sakaguchi *et al.* (34) and has also been obtained from culture filtrates of *Aspergillus fumigatus* [Birkinshaw *et al.* (35)].

Tropolones

In 1942 Birkinshaw *et al.* (36) obtained from the culture fluid of *Penicillium stipitatum* a new acid $C_8H_6O_5$ which was named stipitatic acid. A careful examination of the chemical properties did not lead to any definite conclusions as to its structure although it was readily converted on fusion with potassium hydroxide to 5-hydroxyisophthalic acid. The acid showed certain similarities in general behaviour to puberulic acid, $C_8H_6O_6$, a metabolic product of *Penicillium puberulum* and of *P. aurantio-virens* [Birkinshaw & Raistrick (37)], and it was conjectured that the two acids might belong to a class of compounds hitherto not encountered among mold metabolic products.

Dewar (38) in 1945 first suggested the tropolone concept to account for the recorded chemical behaviour of stipitatic acid. He based the constitution on hydroxycycloheptatrienone (tropolone), at that time unknown, and postulated that stipitatic acid was derived from it by the introduction of hydroxyl and carboxyl groups as substituents. He concluded that the properties of stipitatic acid were accounted for by the formula V and that tropolone IV represents a new type of aromatic structure. Both conclusions have been abundantly justified by further work and syntheses.

In each case the tautomeric formula obtained by interchange of the vicinal =O and —OH groups is of equal probability. It was left to Corbett *et al.* (39, 40) to provide conclusive experimental evidence for the structure of stipitatic acid and for the closely related puberulic acid. They found that the oxidation of stipitatic acid with alkaline hydrogen peroxide gave rise to a mixture of aconitic and malonic acids. Following Dewar (41) they regarded puberulic acid as a hydroxystipitatic acid and adopted formula VI, since this vicinal arrangment of the oxygen atoms is the only one consistent with the production of aconitic acid by alkaline hydrogen peroxide oxidation, observed also in the case of puberulic acid. Quite apart from its value in the

elucidation of the structure of stipitatic and puberulic acids this reaction for conversion of these acids to aconitic acid may be of considerable significance in metabolism since, if it could be effected by the organism, it would provide a means of entry of the tropolones into the citric acid cycle. The constitution of stipitatic acid has now received further confirmation by synthesis [Bartels-Keith *et al.* (42)]. Puberulonic acid, $C_9H_4O_7$, which was first isolated along with puberulic acid from *P. puberulum* (37) is readily converted in hot aqueous solution to puberulic acid with loss of carbon dioxide: $C_9H_4O_7+H_2O \rightarrow C_8H_6O_6+CO_2$ [Corbett *et al.* (40)]. The structure now favoured for puberulonic acid VII, again that of a tropolone, was first proposed by Aulin-Erdtman (43).

Other tropolones occur in nature. The work of Erdtman and his collaborators (44) has identified the α-, β-, and γ- thujaplicins obtained from the conifer *Thuja plicata* as the corresponding α-, β-, and γ- isopropyltropolones. Colchicine and purpurogallin have now been shown to incorporate a tropolone ring in their structure. Recent studies undertaken in several laboratories have thrown much light on the chemistry of this new class of seven-membered ring systems. [See review by Cook & Loudon (45).]

Aromatic Series

Resorcinol derivatives.—The mold *Penicillium brevi-compactum* is known to yield a series of metabolic products, the constitution of which is based on that of resorcinol. These have the general structure VIII where R is (*a*) —CHOH—CO—CH_3, (*b*) —CH_2—CO—CH_3, (*c*)—CO—CO—CH_3+H_2O, (*d*) —COOH (3:5-dihydroxyphthalic acid) [Raistrick and collaborators (46, 47)]. Another more complex product of the same organism, mycophenolic acid, belongs (48, 49), to the same basic series since it most probably possesses the structure IX, the only remaining uncertainties in this structure being slight doubts as to which of the two nuclear hydroxyls is methylated and as to the relative positions of the CO and CH_2 in the lactone ring. Two other fungal metabolic products, clavatol and sorbicillin, belonging to the resorcinol group, show a close relationship. Clavatol (X) was isolated by Bergel *et al.* (50) from culture filtrates of *Aspergillus clavatus*. Its structure was determined by Hassall & Todd (51) who also succeeded in synthesizing it. Sorbicillin (XI) was isolated by Cram (52) from commercial clinical penicillin in which it was present as an impurity. It is therefore derived from *Penicillium notatum*. Its structure differs from that of clavatol only in regard to the ketonic side chain.

An interesting pair of compounds based on the resorcinol structure has recently been investigated. The substances are obtained from the culture filtrates of members of the *P. cyclopium* series of the section *Asymmetrica* sub-section *Fasciculata* of the genus *Penicillium*, which give rise to a number of products. *P. puberulum* affords penicillic acid, first isolated by Alsberg & Black (53). Birkinshaw *et al.* (54) found that the acid was produced in larger yield by *P. cyclopium* and determined its structure. Puberulic and

puberulonic acids (see pp. 375–76) were also isolated from Alsberg & Black's strain of *P. puberulum*.

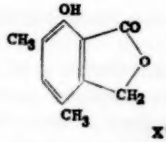
Further examination of strains of *P. cyclopium* [Birkinshaw *et al.* (55)] has brought to light two strains which produce new metabolic products named cyclopolic and cyclopaldic acid. The constitutions of these closely related substances have been investigated and found to be as shown (XII and XIII). The tautomerism of cyclopaldic acid is postulated to account for the reactions, some of which are more consistent with the open form XIIIa and others with the bicyclic hydroxyphthalide form XIIIb. Cyclopolic acid is smoothly converted into cyclopaldic acid by warming with potassium periodate in dilute sulphuric acid.

Another series of the subsection *Fasciculata* of the section *Asymmetrica* of the *Penicillia* is the *P. gladioli* series which is at present represented by a single species, *P. gladioli* Machacek [Raper & Thom, p. 474 (56)]. From strains of this species Brian *et al.* (57) obtained a new product gladiolic acid $C_{11}H_{10}O_5$ which has now been shown to be best represented by the tautomeric formula XV [Grove (58)]. On a medium in which the constituents differed quantitatively but not qualitatively from those employed by Brian *et al.* and with a descendant of the same strain of *P. gladioli*, Raistrick & Ross (59) found in place of gladiolic acid a new crystalline product $C_{11}H_{12}O_5$ which was named dihydrogladiolic acid. When the medium of Brian *et al.* was used both dihydrogladiolic acid and gladiolic acid were obtained in the ratio of about 3.5 parts of the former to 1 of the latter. Grove (58) records the isolation from one particular batch of crude gladiolic acid of a second acid $C_{11}H_{12}O_5$, identical with the dihydrogladiolic acid of Raistrick and Ross and also of the corresponding phthalide (or lactone) $C_{11}H_{10}O_4$. It is possible that the lactone form may not be a true metabolic product but an artefact arising from the method of extraction and purification of the crude gladiolic acid.

Dihydrogladiolic acid is convertible into gladiolic acid by heating with potassium periodate in dilute sulphuric acid exactly as in the oxidation of cyclopolic to cyclopaldic acid. The reverse change is accomplished by reduction of gladiolic acid by heating with iron powder and acetic acid, which yields deoxygladiolic acid (dihydrogladiolide), the lactone of dihydrogladiolic acid.

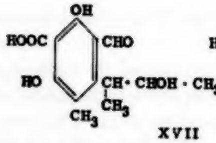
The bicarbonate-insoluble fraction obtained in the purification of crude gladiolic acid was shown by Grove to contain a phenolic substance $C_{10}H_{10}O_3$ which could also be prepared by Clemmensen reduction of gladiolic acid followed by demethylation of the reduction product. Its relation to gladiolic acid thus becomes clear, and it is assigned the formula XVI.

Dihydrogladiolic and gladiolic acids and the phenol XVI are clearly not resorcinol derivatives but are derived from phenol since they possess only one actual or potential nuclear hydroxyl group. They should, therefore, be classed with other mold products possessing this structure such as methyl salicylic acid and mellein. They are considered here, however, since they



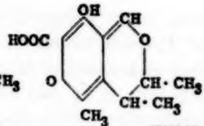
XVI

Phenol from P. gladioli



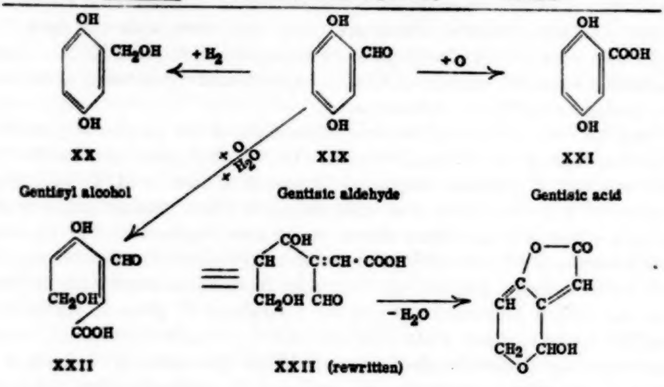
XVII

Citrinin precursor (synthesis)



XVIII

Citrinin



XX Gentisyl alcohol

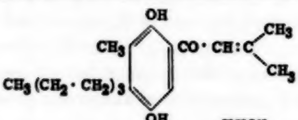
XIX Gentisic aldehyde

XXI Gentisic acid

XXII

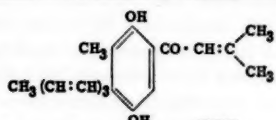
XXII (rewritten)

XXIII Patulin



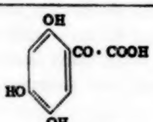
XXIV

Flavoglaucin



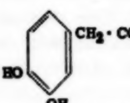
XXV

Auroglaucin



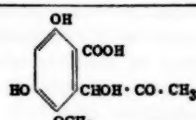
XXVI

Trihydroxyphenyl-glyoxylic acid



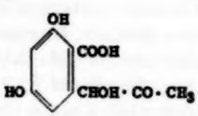
XXVII

Homoprotocatechuic acid



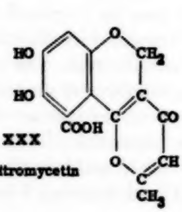
XXVIII

Ustic acid



XXIX

P. brevi-compactum product



XXX

Citromycetin

obviously have such a close affinity with cyclopolic and cyclopaldic acids, differing in fact only in the absence of one phenolic hydroxyl. There is also a close parallel in biological activity between the two pairs of acids. Cyclopaldic and gladiolic acid have a pronounced fungistatic power. Both strongly inhibit the germination of conidia of *Botrytis allii* when tested by the Brian & Hemming technique (60), the former acid being about four times as active as the latter. On the other hand cyclopolic acid and dihydrogladiolic acid show little activity under the same conditions [Smith (61)].

Citrinin, an antibiotic metabolite of *Penicillium citrinum*, *Aspergillus terreus*, and other species, falls into this group. First isolated by Hetherington & Raistrick (62), it was allotted by Coyne *et al.* (63) a structural formula, later modified by Brown *et al.* (64) to the form XVIII. This structure has now been confirmed by total synthesis [Johnson *et al.* (65)]. The phenol XVII which was first synthesised and which is clearly a resorcinol type was converted into (±) -citrinin by cyclisation with sulphuric acid. The racemic form was then resolved to yield the natural (−) citrinin.

Quinol derivatives.—The simplest quinol derivatives obtained from the fungi are gentisyl alcohol XX and gentisic acid XXI, metabolites of *Penicillium patulum* (amongst other fungi) and *Penicillium griseo-fulvum* (66) respectively. Gentisic aldehyde (XIX) has not so far been recorded as a metabolic product of fungi although it is conceivable that it might be the precursor of both the alcohol and the acid, depending on whether the aldehyde could act as hydrogen acceptor or donor in the metabolic environment provided by the particular organism studied. Alternatively the alcohol and acid might be produced simultaneously by a Cannizzaro reaction, with further metabolism of one or the other product and accumulation of the substance not further utilized. The possibility of gentisic aldehyde as a precursor is strengthened by a further consideration: the revised structure for patulin (XXIII) [Woodward & Singh (67)], isolated together with gentisyl alcohol from the culture filtrate of *P. patulum* [Birkinshaw *et al.* (68)] also shows a close relationship to gentisic aldehyde and might conceivably be produced from it by fission of the benzene ring as shown in the hypothetical scheme illustrated by formulae XIX to XXIII, followed by double ring closure.

The break which is assumed to occur in the benzene ring between XIX and XXII is strikingly similar to that which has been postulated in the formation of the intermediate between 3-hydroxyanthranilic acid and quinolinic acid in the scheme proposed for the conversion of tryptophan to quinolinic acid and nicotinic acid (p. 386) The postulated ring closure of XXII to give the pyran structure XXIII resembles that by which the aldehyde XVII forms the second ring in the synthesis of citrinin (XVIII) (p. 377).

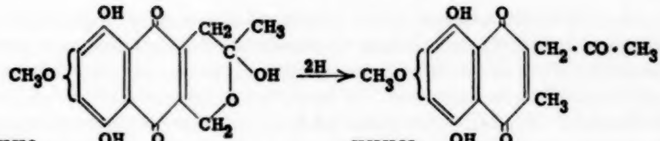
Gould & Raistrick (69) showed that a number of species of the *Aspergillus glaucus* series contained in their mycelium two coloured crystalline metabolites flavoglaucin (XXIV) and auroglaucin (XXV). Their structure was partly elucidated and they were shown by Raistrick *et al.* (70) and Cruickshank *et al.* (71) to possess the quinol structure; the exact position and na-

ture of the substituent side chains was finally established by Quilico *et al.* (72). It is evident from the structural formulae that flavoglaucin and auroglaucin differ only in respect of one side chain attached to the nucleus. This side chain is the n-heptyl group in flavoglaucin and the triply unsaturated n-heptatrienyl group in auroglaucin.

1,2,4-Trihydroxybenzene derivatves.—From the culture fluid of *Polyporus tumulosus* grown on a synthetic medium containing marmite Ralph & Robertson (73) obtained 2,4,5-trihydroxyphenylglyoxylic acid (XXVI), the structure of which was confirmed by synthesis, and another acid homoprotocatechuic acid (XXVII) the only representative so far encountered of the pyrocatechol series, and mentioned here because of its close relationship to the 1,2,4-trihydroxybenzene derivatives. Ustic acid (XXVIII) falls into the 1,2,4-trihydroxy series. It was obtained from the culture filtrate of *Aspergillus ustus* by Raistrick & Stickings (74) when grown on a Raulin-Thom medium. It is closely related to the acid $C_{10}H_{10}O_6$ (XXIX) derived from *Penicillium brevi-compactum* and differs from it in fact only in the possession of an additional (methylated) nuclear hydroxyl group. Another more complex substance of this class is citromycetin (XXX) which is obtained from species of the *P. frequentans* group, some of which were formerly regarded as species of *Citromyces.* Hetherington & Raistrick (75) isolated and characterised the product and determined its partial structure, which was revised and completed by Robertson and colleagues (76, 77). In addition to the benzene nucleus, citromycetin possesses a complete γ-pyrone nucleus and therefore has affinities with kojic acid.

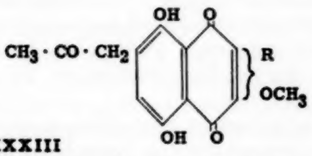
Quinones.—The quinones may be considered as an extension of the quinol series from which they are readily obtainable by oxidation. Indeed in some cases the quinol and its corresponding quinone occur together as metabolic products, as in the case of fumigatin. The culture filtrate of *Penicillium urticae* affords a quinhydrone in the form of dark violet needles. It is composed of three molecules of gentisyl alcohol to one molecule of the corresponding quinone (ω-hydroxytoluquinone) [Engel & Brzeski (78)]. The group of fungal p-toluquinones comprising fumigatin (3-hydroxy-4-methoxy-2,5-toluquinone) and spinulosin (3,6-dihydroxy-4-methoxy-2,5-toluquinone) has now been supplemented by the discovery that 4-methoxy-2,5-toluquinone occurs in cultures of the higher fungi *Coprinus similis* and *Lentinus degener* [Anchel *et al.* (79)].

In the naphthoquinone series Ruelius & Gauhe (80) have obtained from *Fusarium solani* a red pyranoquinone pigment, fusarubin, which diffused into the culture fluid. The constitution XXXI is advanced, in which only the position of the methoxy group remains uncertain. On catalytic reduction with uptake of two molecules of hydrogen and subsequent oxidation in air, fusarubin affords javanicin (XXXII) previously isolated by Arnstein *et al.* (81) and assigned the partial constitution XXXIII ($R=CH_3$) [Arnstein & Cook (82)]. Fusarubin itself shows similar absorption bands to oxyjavanicin of Arnstein & Cook, formulated by them as XXXIII (R=



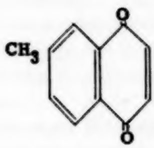
XXXI
Fusarubin

XXXII
Javanicin

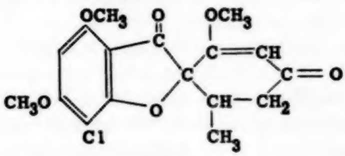


XXXIII

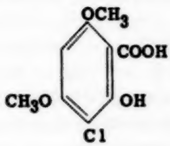
R = CH_3, Javanicin }
R = CH_2OH; Oxyjavanicin } Arnstein & Cook



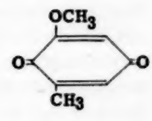
XXXIV
6 - Methylnaphthoquinone



XXXV
Griseofulvin

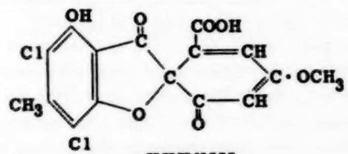


XXXVI



XXXVII

Degradation products of Griseofulvin



XXXVIII
Erdin

CH_2OH). The two substances are probably identical. It is also highly probable that solanione, a pigment obtained from the culture fluid of *F. solani* D_2 purple by Weiss & Nord (83), is identical with javanicin. There is no evidence that fusarubin itself can react in the oxyketo form with two open side chains. The structure of fusarubin (XXXI) is reminiscent of the structure of eleutherin, a pyranoquinone obtained from the tubers of *Eleutherina bulbosa* [Schmid *et al.* (84)].

Another member has now been added to this group of fungal metabolic products by the observation of Bendz (85) that 6-methyl-1,4-naphthoquinone (XXXIV) can be isolated from the metabolism solution of *Marasmius graminum*. The identity of the product was confirmed by synthesis.

In the anthraquinone series further additions have been made to the group of 2-methylpolyhydroxyanthraquinones obtained as fungal metabolic products. ***Penicillium islandicum*** affords a mixture of pigments amongst which islandicin and chrysophanol have been isolated and identified [Howard & Raistrick (86, 87)]. Islandicin, 1,4,5,-trihydroxy-2-methyl anthraquinone bears a close relationship to the isomeric 4,5,8-trihydroxy compound helminthosporin and to the 1,4,5,8-tetrahydroxy compound cynodontin which have been previously described, Chrysophanol, 4,5-dihydroxy-2-methyl anthraquinone is the simplest anthraquinone yet detected as a fungal metabolite and may indeed be regarded as the parent substance from which other fungal anthraquinones can be derived by oxidation and (in some cases) methylation.

Two products isolated from the mycelium of ***Penicillium nalgiovensis*** are of considerable interest, in that they are the first examples of fungal anthraquinones possessing a 3-C instead of a 1-C substituent in the 2- position. Nalgiovensin has the structure *dextro*-4,5-dihydroxy-7-methoxy-2-hydroxypropylanthraquinone in which the nature of the hydroxypropyl group, whether —CHOH—CH_2—CH_3, —CH_2—CHOH—CH_3, or —CH(CH_3)—CH_2OH is undetermined. Nalgiolaxin contains in addition a chloro group and is believed to be *dextro*-(1 or 8)-chloro-4,5-dihydroxy-7-methoxy-2-hydroxypropyl anthraquinone [Raistrick & Ziffer (88)]. It is the first example of a derivative of anthraquinone containing chlorine recorded from any natural source. The optical activity of these products also differentiates them from most anthraquinones derived from natural sources.

Miscellaneous Products

Griseofulvin.—Griseofulvin was first isolated and characterised by Oxford *et al.* (89) who obtained it from the mycelium of ***Penicillium griseofulvum.*** The unique biological activity of a crystalline metabolite of ***P. janczewskii*** towards molds was noted by Brian *et al.* (90, 91), who named the active substance "curling factor," before they were aware of its identity with griseofulvin. The substance causes an unusual stunting and distortion of the germ tubes and hyphae of ***Botrytis allii*** and other fungi. The structural formula proposed by Oxford *et al.* (89) was modified by Grove and McGowan (92) and a new structural formula was finally advanced by Grove *et al.* (93) as a result of extensive chemical degradation and spectroscopic evidence. The structure now adopted is based, as regards potential hydroxyls of the benzene nucleus, on a 1,3,5-arrangement, i.e., the phloroglucinol structure.

Oxidation of griseofulvin with permanganate affords 3-chloro-2-hydroxy-4,6-dimethoxybenzoic acid (XXXVI) whereas chromic acid yields 3-methoxy-2,5-toluquinone (XXXVII). Of the three methoxy groups present in griseofulvin two are, therefore, aromatic methoxyls and the third is derived from an enolic methyl ether; there is no carbomethoxy group. All the carbon atoms present are contained in the two degradation products, none is common to both since the fission of the molecule by sodium methoxide affords

the chloroacid and orcinol monomethyl ether in significant yield. The formation of decarboxygriseofulvic acid $C_{16}H_{19}O_4Cl$ from griseofulvic acid by alkaline hydrolysis is attributed to rupture of the β-diketone system followed by internal Knoevenagel condensation to a dihydrodibenzofuran structure. It is significant that the structure of griseofulvin bears a strong resemblance to one of the alternative structures (XXXVIII) suggested [Calam *et al.* (94)] for erdin, a metabolic product of *A. terreus.*

The number of organic chlorine compounds known to be synthesised by fungi is now such that little surprise is occasioned by the discovery of a new one, although the mechanism by which the chloride ion, the form in which the chlorine is contained in the medium, is converted into the nonionic substituent of an organic molecule remains a mystery. It is believed to be a reaction without parallel in the known reactions of organic chemistry which can be carried out in aqueous solution under physiological conditions of temperature, pH etc.

The finding of the nitro group in fungal metabolites is another instance of the unexpected in nature. It was first observed in chloramphenicol where it occurs as a *p*-nitrophenyl group, but more recently Bush *et al.* (95) have isolated β-nitropropionic acid from an *A. flavus* filtrate. Further instances of the natural occurrence of β-nitropropionic acid are to be found in "hiptagenic acid," a hydrolysis product of the glycosides hiptagin, occurring in the bark of the tree *Hiptage mandoblata*, and of karakin, derived from the berries of the karaka tree *Corynocarpus laevigata*. Hiptagenic acid has been identified as β-nitropropionic acid [Carter & McChesney (96)].

STUDY OF MECHANISM

Applications of Classical Methods

Young preformed felts of *A. niger*, in presence of sodium sulphite and ammonium chloride, produce pyruvic acid and dimethylpyruvic acid [Hida (97)]. Ramachandran & Walker (98, 99) have further investigated this reaction and have found that both acids are formed when D-xylose or glycerol are used as substrate in place of glucose. By the addition of sodium acetate to the glycerol medium in quantities representing a molar ratio of glycerol to acetate of 3:1 to 2:1, the yield of both pyruvic and dimethylpyruvic acids was greatly enhanced. The effect is also produced by addition of ethylene glycol, and, to a less extent, by glycollic acid. Based on these facts a scheme of synthesis of dimethylpyruvic acid is suggested involving the primary condensation of dihydroxyacetone with acetate or glycollate, the reactions with glycollate being represented as in Figure 1.

In the case of ethylene glycol, prior conversion to glycolaldehyde or glycollic acid, followed by the reaction sequence leading to dimethyl pyruvic acid is suggested.

The production of methylheptenone and isobutyl acetate by *Endoconidiophora coerulescens*, detected by Birkinshaw & Morgan (100) may also be

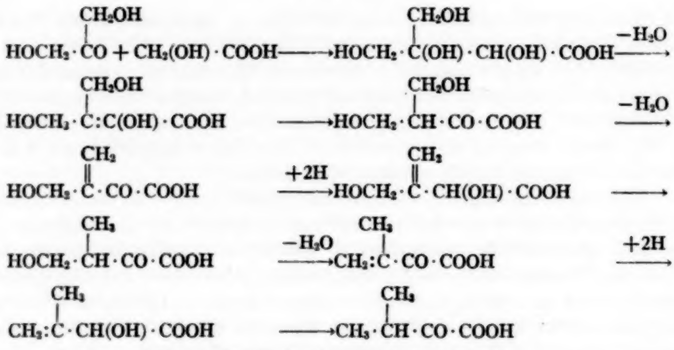


FIG. 1. Synthesis of dimethyl pyruvic acid.

attributable to similar reactions, since triose and glycolaldehyde together with acetone could be regarded as possible ingredients for a methylheptenone synthesis, and isobutyl alcohol might be derived from dimethyl pyruvic acid by decarboxylation and reduction of the resulting aldehyde to the alcohol.

The effect of catalyst poisons, fluoride, iodoacetate, and cyanide on the production of pyruvic and dimethyl pyruvic acid was studied. Only cyanide depressed the yield of the two acids. From this it is argued that pyruvic acid must arise by some mechanism other than production from phosphoglyceric acid as in the Embden-Meyerhof scheme. Walker *et al.* (101) find that when *Aspergillus niger* felts are allowed to act on ethyl acetoacetate or a mixture of this with acetate, in Hida's medium, pyruvic, dimethylpyruvic, and α-ketoglutaric acids are formed together with an unidentified keto acid. The presence of these products was established by paper chromatography. The suggestion is made that the formation of dimethylpyruvic acid from carbohydrate by *A. niger* may proceed by more than one pathway and that its production from acetoacetate may occur by way of a preliminary condensation between acetoacetic acid and acetate yielding a 6-carbon carboxylic acid:

$$CH_3{-}CO{-}CH_2{-}COOH + CH_3{-}COOH \rightarrow CH_3{-}C({=}CH{-}COOH){-}CH_2{-}COOH.$$

This could give rise by decarboxylation to β-methylcrotonic acid or by decarboxylation and reduction to β-methyl-*n*-butyric acid from either of which dimethylpyruvic acid might be derived. The suggested path is reminiscent of the scheme proposed for the syntheses of the carbon skeletons of isovaline and leucine from acetate (see p. 389). Halliwell (102) has found confirmatory evidence for the participation of the tricarboxylic acid cycle in the metabolism of *A. niger* by the simultaneous identification by paper chromatography of oxalic, gluconic, citric, malic, glycollic, succinic, pyruvic, and fumaric acids. The mold is cultured on (*a*) glucose, (*b*) acetate, and (*c*) glucose and

acetate, and after consideration of the order and rate of production and disappearance of the above acids, the conclusion is reached that the initial stages of oxidation of acetate consist of oxalate production, accompanied by a $2C_2 \rightarrow C_4$ condensation to succinate which in turn provides fumaric and malic acid. In presence of glucose and acetate, however, the organism can effect a more rapid and complete metabolism in which the C_4 products are incorporated into citrate, so that less acetate is available for conversion into oxalate.

Use of Mutants

The use of mutants to study the metabolic processes of microorganisms, introduced by the fundamental work of Beadle & Tatum (103) has been pursued with great enthusiasm. A large number of biochemical mutants have been obtained, which include those showing deficiencies for (*a*) seven B-complex vitamins, (*b*) twelve amino acids, (*c*) pyrimidines and purines. The whole subject is reviewed in the textbook of Catcheside (104) who states that

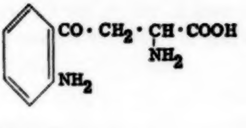
> The number and kinds (of biochemical mutants) found depend in part on the criteria of selection. By the means adopted only lethals are recovered, those affecting non-vital processes not being detected unless they have morphological effects. . . . The biochemical deficiency mutations are by their very nature lethal to the organism which is able to live only if the loss is made up by an external supply of the deficient substance.

Whether these statements are too sweeping must be left to the geneticists to argue [cf. Winge (105)], but it is without question that, even subject to some limitation in the field covered, the method has greatly increased our knowledge of the mechanism of the chemical reactions underlying the vital processes of the fungi.

Most of the fungal studies have been undertaken with *Neurospora*, especially *N. crassa*, the mutants being produced by X-rays, ultraviolet irradiation, or certain chemicals of a toxic nature. Dickey *et al.* (106) drawing attention to the catalysis of peroxide formation by ultraviolet radiation, suggest that organic peroxides may be responsible for the mutagenic effect. The small spontaneous production of adenine-independent mutants by a strain of *Neurospora* which requires adenine for growth is markedly increased by six different peroxides. It is considered possible that peroxides may be involved in the mutagenic action of both x-rays and mustards.

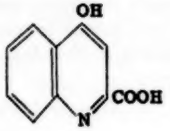
Synthesis of nicotinic acid (*niacin*)—Beadle *et al.* (107) obtained a mutant of *N. crassa* which required either niacin or tryptophan for growth. The growth requirement could also be satisfied by kynurenine (XXXIX), the activity of the DL-form being half that of the L-isomer. In the presence of an excess of kynurenine, niacin was produced. It was conceivable that the pyridine ring in niacin might arise by ring closure of the α-keto acid corresponding with kynurenine to give the naturally occurring kynurenic acid (XL) or, if preceded by oxidation xanthurenic acid (XLI), followed by

oxidation of the benzene ring. The compounds XL and XLI and a series of niacin derivatives were tested by Mitchell & Nyc (108) for growth-promoting or growth-inhibiting properties on the *Neurospora* mutant with negative results.



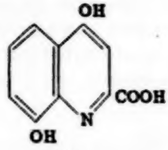
XXXIX

Kynurenine



XL

Kynurenic acid



XLI

Xanthurenic acid

It appeared, therefore, that the pyridine ring does not arise in the manner suggested and also that the insertion of the additional OH in XL to give XLI precedes the formation of the pyridine ring, a possible intermediate being 3-hydroxykynurenine. A consideration of this hypothetical compound suggested the possibility of biological oxidation to give 3-hydroxyanthranilic acid (2-amino-3-hydroxybenzoic acid XLII), a trimethyl derivative of which is found in nature as the alkaloid damascanine. When tested, XLII was found to be highly active in promoting growth of the *Neurospora* mutant. Bonner (109) confirmed by direct comparison the identity of 3-hydroxyanthranilic acid (XLII) with a precursor of nicotinic acid which he had previously shown to be accumulated by another mutant of *Neurospora*. Haskins & Mitchell (110) then found that hydroxykynurenine (XLIII) was a product of tryptophan (XLIV) metabolism and also showed that anthranilic acid (XLV) is formed from tryptophan and may be used for resynthesis of indole (XLVI) and thence of tryptophan, thus completing the cycle.

There was evidence that in rats the excretion of quinolinic acid (carboxynicotinic acid) (XLVII) was increased by administration of tryptophan or 3-hydroxyanthranilic acid [Henderson (111)]. Certain nicotinic-less strains of *Neurospora* were found to grow on high levels of quinolinic acid in place of nicotinic acid, but one which failed to do so accumulated quinolinic acid in quantity. These observations led to the hypothesis that the benzene ring is split in the 3–4 position to yield an unstable aliphatic intermediate (XLVIII) as shown in the general scheme (Figure 2) due to Bonner & Yanofsky (112).

Henderson regards his observations as supporting the hypothesis that quinolinic acid (XLVII) is a true intermediate, but he considers also the possibility that it may result from a ring closure of the true intermediate. The biological activity could then be a result of the reversal of this reaction. Since the activity of quinolinic acid is low on a molar basis, Bonner & Yanofsky (113) suggest that the aliphatic intermediate normally undergoes decarboxylation to XLIX prior to ring closure and that niacin (L) is the immediate product of such closure. If decarboxylation were prevented

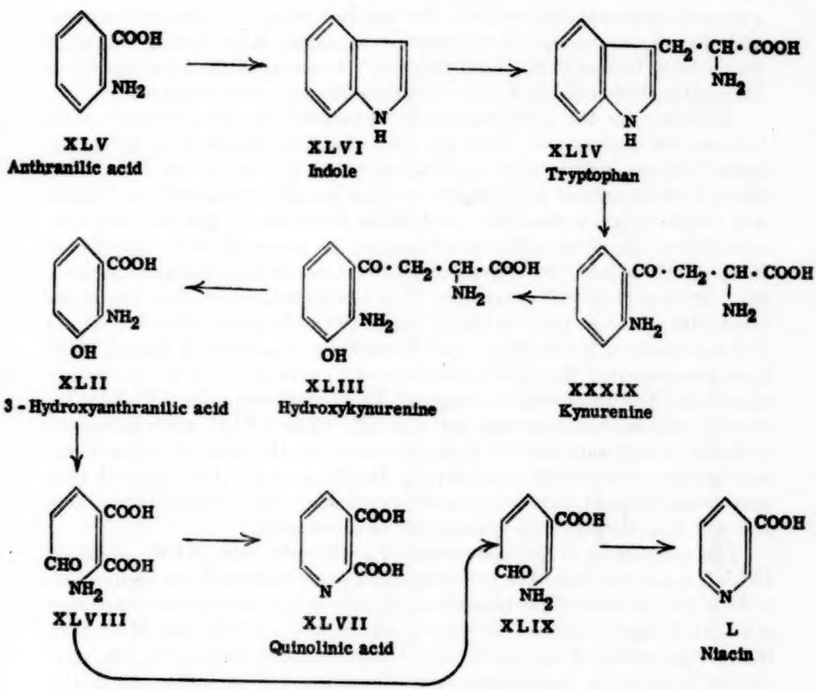


Fig. 2. Biosynthesis of nicotinic acid (niacin).

the aliphatic intermediate (XLVIII) might then undergo ring closure to quinolinic acid. The low biological activity of quinolinic acid would then result from either the reversal of this process, as suggested by Henderson, followed by decarboxylation, or from direct decarboxylation of quinolinic acid in the 2-position.

Leifer *et al.* (114) using N^{15} as tracer observed that approximately 50 per cent of the nitrogen of niacin comes from 3-hydroxyanthranilic acid and the rests comes from the nitrogen of the medium. To account for this they have suggested that a symmetrical diamino compound is intermediate between 3-hydroxyanthranilic acid and niacin. This further complication of the story seems unnecessary however in view of the more critical work by Partridge *et al.* (115), who showed that mutants similar to those used by Leifer *et al.* can synthesise tryptophan under growing conditions. This tryptophan gives rise to additional 3-hydroxyanthranilic acid. Thus the proportion of N^{15} recovered in tracer experiments is primarily a function of the

extent of tryptophan synthesis and is not indicative of a particular mechanism for the conversion of tyrptophan to niacin. With resting mycelium Yanofsky & Bonner (116) found that the N^{15} content of the niacin formed is identical with that of the 3-hydroxyanthranilic acid used as substrate.

Evidence for the participation of kynurenine in the metabolic chain between tryptophan and niacin has been obtained. Beadle *et al.* (107) suggested this possibility when a niacinless strain N-4 of *N. crassa* was discovered which utilized kynurenine, whereas the niacinless strain N-3 which was unable to use kynurenine could utilize 3-hydroxyanthranilic acid. Culture filtrates from strain N-3 could support the growth of N-4. Yanofsky & Bonner (117) isolated the active substance present in these filtrates and identified it as α-N-acetylkynurenine. This compound, which has one seven hundredth of the activity of niacin is thought to be accumulated by strain N-3 as a result of its inability to use kynurenine. Yanofsky & Bonner (116) have demonstrated the rapid conversion of kynurenine to niacin in resting mycelium. The intermediate stage of 3-hydroxykynurenine (XLIII), inserted in the metabolic scheme between XXXIX and XLII, while extremely probable, is still unproven for fungal metabolism. However, by paper chromatographic and enzymic techniques, Dalgliesh *et al.* (118) showed that pyridoxine-deficient rats converted tryptophan into 3-acetylkynurenine and α-N-acetylkynurenine, amongst other substances.

The mechanism of the conversion of anthranilic acid (XLV) to indole (XLVI) is not yet clear. The amino group of anthranilic acid has been shown to form the nitrogen of the indole ring [Bonner & Partridge (119); Partridge *et al.* (115)], and it appears probable from the work of Nyc *et al.* (120) that the carboxy group of anthranilic acid is lost on indole formation. The conversion of indole to tryptophan by condensation with serine is well established [Tatum & Bonner (121)] and indeed the enzyme system responsible for this synthesis can be extracted from wild type *Neurospora* [Umbreit *et al.* (122)]. Pyridoxal phosphate is required as coenzyme. Mitchell & Lein (123) obtained a mutant strain which required tryptophan for growth but which could not utilize indole. Cell-free extracts of this mutant lacked the ability to couple indole and serine to give tryptophan, thus affording direct evidence that the loss of activity of an essential enzyme is attributable to gene mutation.

It now appears that the general metabolic pathway between tryptophan and niacin as outlined for *Neurospora* is common also to many animals including insects, and to higher plants and bacteria and is a pointer to the essential unity of the fundamental processes underlying the biochemistry of all living organisms [cf. review by Dalgliesh (124).]

Biosynthesis of isoleucine and valine.—The investigation of the mechanism of this synthesis began with the observation of Bonner *et al.* (125) that strain 16117 of *N. crassa* required the aminoacids isoleucine (LI) and valine (LII) for growth. Bonner (126) presented evidence that the requirement of the two amino acids was due to the accumulation of an isoleucine

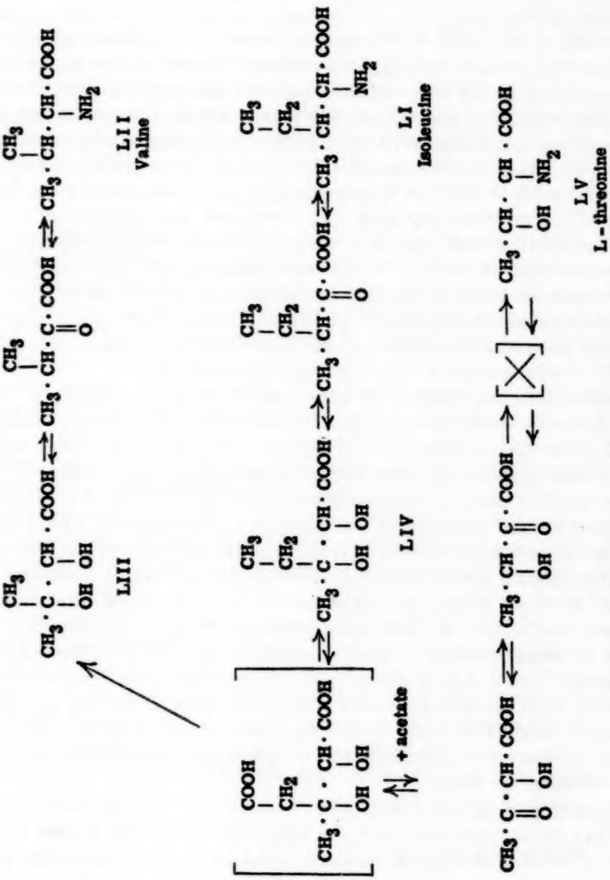


Fig. 3. Biosynthesis of isoleucine and valine.

precursor which prevented conversion of an analogous intermediate into valine, by a species of competitive inhibition. It was suggested that the intermediates involved were the respective α-keto acids corresponding to the two amino acids.

Adelberg *et al.* (127) attempted to isolate α-keto-β-ethylbutyric acid (corresponding with isoleucine) from culture filtrates of the strain 16117. Although this acid was not obtained they did demonstrate that these isoleucine-free filtrates contained considerable amounts of an unknown intermediate which had high activity for various isoleucine-requiring strains of bacteria. By paper chromatography Adelberg & Tatum (128) showed that two organic acids in addition to succinic acid were produced in considerable amount. The faster-moving spot was associated with isoleucine activity the slower-moving spot was due to αβ-dihydroxy-β-methylbutyric acid (LIII) a precursor of valine. The acid corresponding with the faster-moving spot, was isolated in similar manner by Adelberg *et al.* (127) and shown to be αβ-dihydroxy-β-ethylbutyric acid (LIV). Adelberg (129) found that this compound was about five times as active as isoleucine in supporting growth of *E. coli*. It was concluded to be a normal intermediate.

To determine the manner of synthesis of these precursors, *N. crassa* 16117 was grown in the presence of C^{14}-labelled acetate [Tatum & Adelberg (130)]. The precursors were degraded and the radioactivity of the degradation products was determined. The following deductions were made: (*a*) The 4-carbon straight chains of isoleucine and valine are formed at least in part by a "head to tail" coupling of two molecules of acetate; (*b*) The 2-carbon side chain of isoleucine is derived from acetate, the carboxyl of which becomes the terminal methyl group; (*c*) The single-carbon side chain of valine is derived from the methyl carbon of acetate. It appears that two molecules of acetate first couple to form some unknown 4-carbon intermediate; by analogy to citrate formation from oxaoacetate and acetate, this might be predicted to have a β-keto group. The intermediate then couples through its C_3 atom with the methyl of another acetate molecule to form a 6-carbon dicarboxylic acid. The latter then undergoes reduction of the side chain carboxyl to give the carbon skeleton of isoleucine, whilst decarboxylation yields the valine structure.

Umbarger & Adelberg (131) have repeated and reinterpreted previous work. They put forward the scheme of biosynthesis shown in Figure 3 which is in accord with all the data so far established for *N. crassa* and other organisms. The introduction of threonine (LV) in this scheme is in accordance with the work of Teas (132) which showed that threonine and isoleucine are interconvertible through a common 4-carbon precursor. It will be noted that the αβ-dihydroxy acids corresponding to isoleucine and valine are presumed to be converted to their respective keto acids prior to amination.

One step in the indicated method of synthesis of valine is of particular interest, namely the methylation of the 4-C acid, which is accomplished by attachment of acetic acid through its methyl group and subsequent decar-

boxylation. This mechanism under the name of "the acetic acid hypothesis" was considered by Challenger (133) in discussing the methylation of arsenious acid by *Sterigmatocystis brevicaulis* with production of trimethylarsine. No evidence could be obtained in support of the hypothesis in this particular case.

In the work described, employing mutants of *Neurospora crassa*, the chemical methods were in some cases supplemented by those involving the use of radioactive tracers. The application of isotopes for labelling atoms in recent mold investigations is best exemplified in the study of the citrate metabolism of fungi.

Use of Tracers

Citrate metabolism.—In studies on the oxidative metabolism of yeast Weinhouse & collaborators (134, 135) obtained definite evidence in support of the citric acid cycle. The method consisted in determining the distribution of isotopic carbon in citrate formed during the oxidation of acetate labelled with C^{13} in the carboxyl group. It is possible to calculate that any citrate formed through the cycle would have in its two primary carboxyl carbons an average of 75 per cent of the isotope content of the acetate carboxyl, whereas the corresponding value for the single tertiary carboxyl would be 50 per cent. The close correspondence between these theoretical values and those actually found led the authors to conclude that citric acid represents a major metabolic pathway in yeast.

In extending the method to *Aspergillus niger*, Lewis & Weinhouse (136) found that the metabolism of carboxyl-labelled acetate leads to the formation of citric acid labelled in the carboxyl carbons with approximately 1.5 times more isotope in the primary carboxyls than in the tertiary. Methyl-labelled acetate under the same circumstances yields citrate labelled in both carboxyl and noncarboxyl carbons. In the citric acid cycle the acetate methyl carbons are not lost in the first round of the cycle. They first appear as central carbons of citrate and only after getting into the carboxyls of C-4 acids are they liberated as CO_2 in subsequent rounds of the cycle. By following these methyl carbons through successive cycles one finds that a steady state is rapidly reached when the C-4 acids have three of four carbons labelled, the

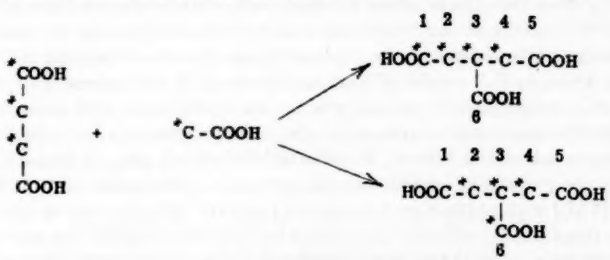


Fig. 4. Citrate metabolism.

unlabelled one being a carboxyl carbon. Further condensation with methyl-labelled acetate yields two varieties of citrate, depending on whether condensation occurs on the carbon adjacent to or on carbon removed from the labelled carboxyl carbon (Figure 4).

It can be calculated that, assuming a value of 100 for overall activity of the six citrate carbons, the noncarboxyl carbons would have equal activities of 150, the primary carboxyls 38, and the tertiary carboxyl 75. The observed values were in good agreement with the calculated in two respects. (*a*) The activities of the three noncarboxyl carbons were essentially equal. (*b*) The proportion of activity in these three carbons was almost exactly the calculated value. The agreement for the primary and tertiary carboxyls was not so good, the former being too high and the latter too low. This may indicate that supplementary mechanisms are in operation. The interpretation is further complicated by the migration of the methyl-carbon to the carboxyl position in the methyl-labelled acetate, which occurs to an appreciable extent, although not sufficiently to be a major reaction. The conclusion is drawn that the citric acid cycle is a major pathway for acetate utilisation and citrate formation by this organism.

Lewis & Weinhouse (137) further examined the assimilation of $C^{14}O_2$ in oxalate and citrate by *A. niger*. Acetate and glucose were employed as substrates for oxalate and citrate formation. In all experiments, labelled carbon was found in both citrate and oxalate, and to a small extent in acetate but not in formate. A study of the distribution of the C^{14} in the citrate carbons showed the primary and tertiary carboxyls to have almost equal activity, in agreement with the results of Martin *et al.* (138). When the distribution of C^{14} in the primary carboxyls of some of these labelled citrates was examined by the method of Potter & Heidelberger (139) it was found that when the citrate was degraded enzymically to α-ketoglutarate the preponderance of activity (85 per cent) was in the carboxyl adjacent to the keto group. These results are interpreted to mean that the carboxylation of pyruvate represents a major if not exclusive, pathway of CO_2 assimilation in *A. niger*.

Radioactive oxalate was obtained in every experiment, although of lower activity than the citrate. Since labelled oxaloacetate derived from pyruvate and $C^{14}O_2$ by the Wood-Werkman reaction should give rise to unlabelled oxalate and labelled acetate by central fission, this would appear to exclude oxaloacetate as the source of oxalate. However, if we assume that the C^{14} can become randomised through the known equilibrium with fumaric acid, a perfectly reasonable assumption, then oxalacetate can be considered as the source not only of citrate, but also of labelled oxalate and acetate. There is also the possibility of CO_2 fixation by way of the malic enzyme [Ochoa *et al.* (140)] with obligatory formation of malate, which is one of the stages in the fumarate equilibrium and would explain more readily the randomisation. De Baun *et al.* (141), from a study of the degradation of α-ketoglutaric acid by wood-destroying fungi also concluded that the scheme α-ketogluta-

rate→succinate→fumarate→malate→oxalate was applicable in the case of *Lentinus lepideus*.

The hypothesis of Lynen & Lynen (142) that oxalate may also arise by a fission of oxalosuccinic acid is not supported by the experimental findings, neither is the direct oxidation of acetate to oxalate, as suggested by Challenger *et al.* (143) and later by Nord & Vitucci (144), although the latter reaction may occur to a minor extent.

Foster and his collaborators (145) have studied radioactive CO_2 assimilation by *Rhizopus nigricans* and have found definite evidence for the dehydrogenative coupling of two acetates to yield succinate (Thunberg reaction). Foster & Carson (146) have observed that in *Aspergillus niger* methyl-labelled acetate affords citrate equally labelled in the noncarboxyl carbons and conclude that citrate arises by successive C_2 condensations: $2C_2 \rightarrow C_4$; $C_2 + C_4 \rightarrow C_6$. To account for the appearance of acetate methyl-carbon in citrate carboxyls a very active respiratory cycle is assumed. This phenomenon however can alternatively be ascribed to the functioning of the citric acid cycle as already shown.

The conclusion of Lewis & Weinhouse (136) seems reasonable. It envisages a condensation of acetate with oxaloacetate supplied from three sources: (*a*) the citric acid cycle (Figure 5), (*b*) the Wood-Werkman reaction;

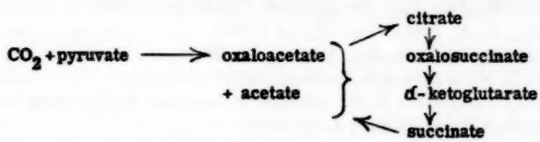


Fig. 5. Citric acid cycle.

(*c*) the Thunberg condensation. The extent to which these processes occur would be expected to vary under different conditions. Citric acid is regarded, not as an end product of normal metabolism, but as a normal intermediate which may accumulate under certain circumstances because of some unknown interference in its further metabolism.

Carson *et al.* (147) using a stepwise degradation method of Mosbach *et al.* (148) determined individually the isotopic content of each of the six carbon atoms of citric acid synthesised by *A. niger* in presence of $C^{14}H_3 \cdot COOH$ and $C^{13}O_2$. The finding of appreciable C^{14} activity in the 5-carboxyl indicated the existence of reactions leading to formation of doubly-labelled acetate. CO_2 fixation or exchange reactions were confirmed by finding excess C^{13} (from $C^{13}O_2$) in the citrate carboxyls. The results confirm the existence of extensive C_4- or C_6-acid cycling during biosynthesis. Wide divergence in the activity of the primary carboxyls confirms the Ogston concept (149).

INDUSTRIAL MOLD FERMENTATIONS

The consideration of industrial applications of mold fermentations, which are obviously of great importance, can receive only the briefest mention

here. In this sphere the most outstanding recent developments may be grouped under three heads: (*a*) the commercial production of antibiotics and riboflavin; (*b*) the ever increasing use of submerged fermentations, which has led to an intensified study of the particular problems arising in this type of fermentation and of the modifications necessary in the conversion of existing surface culture processes to submerged culture, applying particularly to the citric acid fermentation; (*c*) the closer investigation of the production of known fungal metabolites of potential importance in industry. This usually takes the course of improvement of yield in laboratory studies followed by transference to pilot plant (or larger) scale. Such substances as itaconic and fumaric acids have been investigated in this way.

The annual articles of Lee *et al.* (150, 151, 152) provide a valuable review and documentation of progress in the whole field of industrial fermentations.

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RUMINANT NUTRITION[1,2]

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Since the last general review of ruminant nutrition by Marston (1) in 1939, there has been a marked trend toward grassland agriculture as a means of conserving and improving the soil. The economical production of milk, meat, and wool by ruminants is based on use of pasture, hay, and silage crops.

The relation of soil and plant deficiencies and toxic constituents in soils to animal nutrition were reviewed by Maynard (2). Nutritional deficiencies in farm mammals on natural feeds were reviewed by Huffman & Duncan (3). Summaries covering ruminant nutrition with emphasis on nutritive requirements have been made by Loosli *et al.* (4), Guilbert *et al.* (5), and Pearson *et al.* (6). The effect of different methods of forage crop conservation on nutritive value, calf nutrition, interrelationship of nutrition and hormones, and the role of vitamins in the nutrition of ruminants will not be reviewed because of limited space. This brief review is concerned primarily with the composition and nutritive value of roughages for ruminants with special emphasis on the role of microbial digestion and synthesis. The nutrition of the ruminant may be primarily a question of balancing the nutrients required by digestive tract microbes.

The Fermentation Vat

Herbage is characterized by a high proportion of celluloses and hemicelluloses, which cannot be digested by the enzymes in the digestive juices secreted by ruminants, and by considerable amounts of nonprotein nitrogen compounds which are of little value until synthesized into proteins by rumen microorganisms. The ruminant, however, is equipped with a spacious fermentation vat (omasum, reticulum, rumen) which is well suited to the maintenance of a large microbial population capable of digesting plant constituents and synthesizing many nutritious compounds for the host. In addition to its large size, the fermentation chamber has an added advantage of location at the anterior end of the digestive tract which gives the microorganisms access to the readily available nutrients consumed by the host. This location results in the addition of large amounts of saliva which transport certain nutrients from the body of the animal to the fermentation vat. The continuous flow of saliva also aids in maintaining a high water level in the rumen to promote fermentation. The transition of the calf to a functional ruminant appears to be at from four to six weeks of age, as indicated by the development of the characteristic rumen odor [Kesler *et al.* (7)] and by a

[1] The survey of the literature pertaining to this review was concluded in September, 1952.

[2] The following abbreviations are used: N. F. E. for nitrogen-free extract; T. D. N. for total digestible nutrients.

decline in blood glucose [McCandless & Dye (8); Crane & Hansen (9)].

New methods of studying rumen digestion, *in vitro*, have been reported which allow greater control and more extensive observations under experimental conditions than do the methods *in vivo*. Marston (10) devised a method for regulating the pH, maintaining the temperature at 39°C., and maintaining anaerobiosis by passing nitrogen through the system continuously. Louw *et al.* (11) reported a method for removing the lower fatty acids formed during digestion by suspending a semipermeable sac in a large volume of growth medium so the acids could dialyze out of the field of fermentation. This system increased the rate of digestion of cellulose in the semipermeable sac over that in the all-glass container. Gall & Glaws (12) reported that the Gram stains and culturability of the microorganisms obtained from the semipermeable system compared favorably with those found in the original rumen contents. The impermeable system gave poor results.

The physiology and biochemistry of rumination have been reviewed by Owen (13), and ruminant digestion was reviewed by Elsden & Phillipson (14).

Rumen microbiology.—Splendid reviews of this topic have been made by Sijpesteijn (15) and Hungate (16). The complexity of rumen bacteriological studies was shown by the findings of Bryant (17), who reported that 51 different kinds of bacteria had been isolated from the rumen of cattle on a single ration. Of the 102 cultures isolated, 101 were anaerobic, 28 were motile, 5 produced hydrogen sulfide, 26 liquified gelatin, 15 digested cellulose rapidly, 43 hydrolyzed starch, 76 produced acid from glucose, 69 produced acid from cellobiose, and 40 produced acid from xylose.

Pure cultures of bacteria from the rumen have been isolated by several investigators. Hungate (16) isolated several strains of non-spore-forming rods and colorless and yellow cocci that digest cellulose. Gall & Huhtanen (18) and Gall *et al.* (19) made about 5,000 isolations of bacteria from the rumina of about 350 cattle and sheep from several herds and flocks in three states. Five criteria were used for judging true rumen bacteria: (*a*) anaerobiosis; (*b*) presence in numbers of one million or more per gm. of fresh rumen contents; (*c*) isolation of a similar type of bacterium at least ten times from at least two animals; (*d*) isolation from animals in at least two geographical locations; and (*e*) production by the organism of end-products found in the rumen. Anaerobiosis was considered to be the most important criterion for judging the bacteria to be true rumen organisms. Direct slide counts of the bacteria indicated about 50 to 100 billion bacteria per gm. of fresh rumen contents. Gall & Huhtanen (18) isolated 20 types of rumen bacteria. Five of these were described and named. These investigators recently described improved techniques for isolating and purifying rumen microorganisms (20). Sijpesteijn (15, 21) also isolated a cellulose-digesting strain of bacteria in pure culture from the rumen.

One of the first attempts to classify rumen bacteria into groups was

made by Pounden & Hibbs (22, 23) who divided the rumen bacteria into two groups: the hay flora and the grain flora. The hay flora were subdivided into (*a*) large Gram-positive coccoids in closely knit pairs and (*b*) large Gram-positive, thick, fairly square rods, and smaller Gram-negative short rods in fours and multiples of four. The grain flora consisted of medium sized, comparatively thin, Gram-positive, and Gram-negative rods resembling the coliform organism. Calves isolated from older cattle shortly after birth failed to develop the typical hay flora unless inoculated with cud material from the rumina of cattle with normal hay flora.

Moir & Masson (24) proposed a scheme based on direct microscopy for the microscopic identification of rumen bacteria commonly encountered in sheep. The terms employed to describe 33 forms fall into the following categories: (*a*) morphological; (*b*) histochemical and staining reactions; and (*c*) ecological and functional. These investigators acknowledged that their scheme is not final but should provide a starting point for continued research in the microscopic examination and description of the rumen microflora. Modifications of techniques used in processing rumen samples for bacteriological studies were suggested by Doetsch *et al.* (25).

Tympany (*Bloat*).—Ruminants pastured on immature legumes are prone to develop tympany through failure to eructate gas formed during rumen fermentation. Two types of bloat have been established: (*a*) an excessive accumulation of free gas and (*b*) an excessive accumulation of gas held in a "frothy" emulsion. The latter type of bloat is effectively treated with antifrothing agents [Clark (26)].

Some of the theories explaining bloat have been reviewed by Cole *et al.* (27). The most commonly accepted theory applicable to accumulations of free gas in the rumen is that of "physical deficiency" or "lack of coarse roughage." According to this theory, most bloat on green legumes results from the absence of a scabrous material which is necessary to stimulate belching. The theory is supported by the fact that the incidence of bloat can be reduced by using pasture mixtures containing less than 50 per cent legumes. Ruminants that eat considerable amounts of hay before being allowed to graze legumes are less likely to bloat. This theory does not explain the "frothy" type of bloat, however, nor has it been proved that the stimulation of nerve fibers in the esophagus or rumen elicits the act of eructation. Belching is facilitated by the administration of turpentine, but the exact mode of action has not been determined.

The "toxic substance" theory of bloat has been advanced by Shanks (28) and Kerr & Lamont (29), who postulated that bloat is due to a form of anaphylactic shock since the injection of epinephrine into "bloaty" animals brought about an immediate cure. They explained that such shock results in a spasm of the smooth muscle in the cardiac sphincter, whereby gas produced in the rumen is unable to escape. Ferguson *et al.* (30) developed a technique for testing *in vitro*, on the smooth muscle of the rabbit intestine, plant juices from normal and "bloaty" pastures. Plant juice from "bloaty"

pastures contained a muscle-inhibiting compound which may be connected with bloat. These investigators (31) isolated the compound tricin, a flavone from alfalfa, which had the same effect on smooth muscle as plant juice from "bloaty" pastures. Epinephrine counteracted this effect. The compound has not been used to produce bloat in the intact ruminant. Additional evidence which supports the toxic substance theory was provided by Parsons *et al.* (32) who reported the production of bloat in sheep by drenching with juice prepared from legumes from the field where the animals were being pastured. Juice from grass had no effect.

Carbohydrates

Carbohydrates in herbage are the most important sources of energy for ruminants. A summative analysis of timothy hay by Flanders (33) showed that 52 per cent of the dry matter consists of celluloses and hemicelluloses and 11.6 per cent of lignin. The various kinds of carbohydrates in forage are numerous and not precisely defined. Lignin is discussed with carbohydrates because of its close association in plant structure. In the conventional feed stuff analysis, carbohydrates are found in crude fiber and nitrogen-free extract (N. F. E.) fractions. The N. F. E.[2] is determined by difference.

Crude fiber and N. F. E.—The crude fiber fraction which makes up about 30 per cent of the dry matter of herbage is not a single compound, but, according to Norman (34), consists of celluloses, lignins, and hemicelluloses. These constituents occur in different ratios in various plant species. Nordfeldt *et al.* (35) determined the composition of the crude fiber of hay on the percentage basis as follows: cellulose, 80.1; lignin, 11.5; pentosans (calculated as 50 per cent xylan and 50 per cent araban), 10.9; and a small amount of crude protein and ash. The composition of the N. F. E. fraction was: cellulose, 20.5; lignin, 9.7; and pentosans, 29.4 per cent. These three constituents accounted for 59.7 per cent of the N. F. E. The remaining 40.3 per cent consisted of sugars, hexosans, organic acids, etc. The crude fiber in the hay contained 40.6 per cent of the lignin and the remaining 59.4 per cent appeared in the N. F. E. Of the total pentosans in the hay, 17.7 per cent were in the crude fiber and 82.3 per cent in the N. F. E. Cellulose was distributed as follows: 69.3 per cent in the crude fiber and 30.7 per cent in the N. F. E. Thus, as much as 59.7 per cent of the N. F. E. of hay may consist of lignin, cellulose, and pentosans; the remaining 40.3 per cent consists of sugars, hexosans, organic acids, etc. It is of interest that Nordfeldt *et al.* found 28.5 per cent of the lignin in oat straw in the crude fiber and 71.5 per cent in the N. F. E. fraction, although the total lignin content of the straw was greater than that of hay. The N. F. E. of the oat straw contained 78.8 per cent of total pentosans.

From data on limited digestion trials with sheep, Nordfeldt *et al.* (35) observed that during digestion a part of the lignin moved from the crude fiber to the N. F. E. fraction. Celluloses, pentosans, and lignin made up 90

to 95 per cent of the N. F. E.[2] in the feces, but only 54.3 of that in the hay. The shift of lignin from the crude fiber to the N. F. E. fraction during passage through the digestive tract may explain the report of Crampton & Maynard (36), using the data supplied by Morrison (37), that in dry roughages, 39 per cent of the samples showed as great or greater coefficients of digestibility of crude fiber than the N. F. E. A large percentage of the pentosans and lignin was found in the N. F. E. in both plants and feces, while most of the hexosans were found in the crude fiber in both plants and feces, according to Bondi & Myer (38). The coefficients of digestibility of the total pentosans in the plant species studied varied from 60 to 79 and in the N. F. E. from 53 to 88. The digestibility by sheep of crude fiber and its pentosan and cellulose components showed wide variations with different feeds and was inversely proportional to the lignin content [Nauman (39)]. Lignin was indigestible in nearly all cases.

The value of crude fiber and the N. F. E. fractions as criteria of the nutritive value of roughages for ruminants has been questioned by many investigators. Partition of the carbohydrate portion of the feed into cellulose, lignin, and other carbohydrates was suggested by Crampton & Maynard (36). This type of partition of herbage has also been suggested by Ellis *et al.* (40). The possibility of using holocellulose as an indication of the carbohydrate fraction has been suggested by the work of Adams & Catagne (41) and Flanders (42). Ferguson (43), however, concluded that the determination of holocellulose is unlikely to be of much value in herbage analysis because of the difficulty of removing all of the lignin, protein, and ash.

Stage of maturity.—Of the many factors which affect the chemical composition and digestibility of herbage, the most important is the stage of maturity. Changes in the composition of timothy tops were studied by Phillips *et al.* (44) who reported an increase in crude cellulose, pentosans, lignin, total methoxyl, and the methoxyl content of the ash-free lignin. An increase in crude fiber and lignin occurred in timothy and red clover, according to Hvidsten *et al.* (45). Perennial rye and alfalfa from early to late stages of growth were studied by Armstrong *et al.* (46) who found that crude fiber, lignin, and cellulose increased with maturity. According to Patton & Gieseker (47), who studied five species of grass with advance in maturity, the increase in lignin closely paralleled the increase in cellulose. Louw (48) determined the chemical composition and digestibility of a mixture of pasture grasses taken at four stages of growth with sheep. The amount of natural cellulose increased from 44.2 to 49.5 per cent between the first and the last harvest, while the digestibility coefficient decreased from 75 to 58. The natural cellulose was composed of true cellulose and xylan. The digestibility of the true cellulose decreased from 76 to 61 and the xylan from 72 to 56. The lignin increased from 9.9 to 11.4 per cent and the digestibility declined from 24 to 12 per cent. The amount of hemicelluloses in the herbage did not change significantly, but the digestibility decreased from 54 to 44 per cent. Of the two constituents into which the hemicelluloses were divided, uronic acids

and pentosans, the former decreased more rapidly in digestibility than the latter. A lignin-hemicellulose complex and a complex between lignin and xylan were suggested.

The changes in chemical composition and digestibility of the brome plant from 10.2 to 91.4 cm. in height was investigated by Sotola (49). The crude fiber increased from 19.8 to 35.9 per cent. The dry matter was 81 per cent digestible at the 10.2 cm. stage and only 56 per cent digestible at the 91.4 cm. stage. The digestibility of crude fiber and N. F. E.[2] followed similar trends. The changes in chemical composition and in digestibility of orchard grass from the pasture stage to the mature hay stage were investigated by Ely (50). The amount of crude fiber increased from 26.9 to 35.0 per cent and the N. F. E. from 35.0 to 41.2 per cent, while the coefficients of digestibility decreased as follows: crude fiber from 81 to 68 and N. F. E. from 79 to 72. Changes in the composition of pasture herbage (predominantly timothy) from the vegetative to full bloom stage were investigated by Richards & Reid (51) who reported: methoxyl, 1.58 to 2.10; lignin, 5.8 to 8.6; crude fiber, 38.1 to 33.0; and N. F. E. 33.5 to 47.9 per cent. The changes in the coefficients of digestibility, as determined with steers, were: methoxyl, 55 to 31; lignin, −14 to −9; crude fiber, 73 to 58; and N. F. E., 79 to 60.

Microbial digestion.—The early work on microbial digestion of carbohydrates was reviewed by Elsden & Phillipson (14) and Baker & Harriss (52).

In sheep which were fed a constant ration of alfalfa hay and wheat straw before slaughter, Gray (53) found, upon post-mortem examination by use of lignin ratios, that 70 per cent of the cellulose was digested in the rumen, 17 per cent in the cecum, and 13 per cent in the colon.

A low dry-matter content of rumen ingesta favored a rapid breakdown of cellulose [Balch (54)]. The rate of breakdown of dry matter was faster in the ventral than in the dorsal sac of the rumen. Miles (55) also reported that the digestion of dry matter, cellulose, and pentosans of alfalfa hay was more complete in the ventral portion of the rumen. Lignin was only slightly digested.

The carbohydrate fraction of roughages at the stage of maturity used as hay and pasture is associated with a lignin complex in a form which impedes efficient digestibility. It is of interest that the work of Lawton *et al.* (56) showed that irradiation of basswood with high velocity electrons altered the structure in such a way that some of the insoluble carbohydrate compounds became available to rumen bacteria. Using the cotton thread technique, Hoflund *et al.* (57) concluded that there was no significant difference between digestion of cellulose, *in vivo* and *in vitro*. Rumen microorganisms appear somewhat sensitive to the level of sugar in the ration, according to Hoflund *et al.*, who found that the concentration of glucose for optimum cellulose digestion *in vitro* varied between 0.1 and 0.2 per cent. Higher concentrations depressed cellulose digestion. In the intact sheep, cellulose digestion was stimulated at 1 and 3 per cent levels of sucrose feeding, while

a 9 per cent level depressed digestion. Louw *et al.* (58) also found that cellulose digestion was depressed by supplementing a poor grass hay with a mixture which contained starch, casein, dried brewers yeast, and a complete mineral mixture. The addition of 200 gm. of green (fresh) alfalfa daily to the ration improved cellulose digestibility, but the greater digestibility of the cellulose could not be attributed to the cellulose in the green alfalfa, as the amount introduced into the ration was too small. Cud-inoculated calves digested a higher percentage of the cellulose and dry matter than uninoculated calves and consumed more dry matter [Conrad *et al.* (59)].

The effect of fasting on the rate of cellulose digestion of rumen contents was studied by Quin *et al.* (60) who used sheep that were fed grass hay. The rate of digestion was not affected after 24 hours of fasting, but was markedly retarded after 48 hours. The normal rate of cellulose digestion was restored, however, within 24 hours after feeding had been resumed. Similar results were obtained with alfalfa hay as a source of natural cellulose, except that recovery was delayed up to about 10 days after feeding was resumed. The loss of power to digest cellulose in the rumen during starvation is due either to the diappearance of cellulolytic microorganisms or the inability of the organisms to attack cellulose in the absence of other essential nutrients. Similar results were reported by Coop (61) who found that the microfloral activity of starved sheep rapidly decreased to a low level as was indicated by volatile fatty acid production and pH of rumen contents.

Evidence that the level of protein in the ration influences the digestion of cellulose was reported by Hoflund *et al.* (57). The supplementation of a hay ration of sheep with 20 gm. of casein and 200 gm. of molasses did not depress cellulose digestion, but cellulose digestibility declined markedly when the casein was discontinued.

In a continuation of the work of Hoflund *et al.* (57), Clark & Quin (62) showed that sheep fed a ration of poor grass hay alone declined in appetite and body weight, but when either urea and molasses or 200 gm. of alfalfa hay daily were added as supplements, the intake of hay increased and so also the body weight. Sheep fed sodium nitrate as a partial source of nitrogen showed an increase in dry matter consumption and a decreased loss of body weight. Apparently nitrate can be safely and advantageously substituted for urea as a partial source of nitrogen, provided sufficient sugar is given simultaneously. Sodium nitrate up to 4 per cent and molasses, 12 per cent by weight, added to the hay, resulted in accelerated cellulose digestion. The beneficial effects were not only due to the extra nutrients derived from the supplements, but also to an acceleration of cellulose digestion which resulted in an increased consumption of hay. The percentage of cellulose digested was not influenced. It is well known that the forestomachs of ruminants are organized to exclude the passage of coarse fibrous particules into the abomasum. It must, therefore, follow that a retardation of cellulose digestion will necessitate a more protracted sojourn of the feed in the rumen and a corresponding decrease in the total amount of bulk handled within a

given time. It has been pointed out previously that nutrients are supplied more cheaply in the form of herbage than as concentrates.

The ability of rumen microorganisms, obtained from a steer fed poor quality hay, to use urea and digest cellulose was investigated by Bentley *et al.* (63) by the *in vitro* technique of Burroughs *et al.* (64). Ammonia (urea) utilization declined 25 per cent within one week, and the rate of cellulose digestion declined 90 per cent during the first four weeks of poor hay feeding. Supplementation of the ration with a mineral mixture restored the rumen microorganisms to normal activity.

Many investigators have shown the need of a readily available carbohydrate for the efficient conversion of urea nitrogen to protein. Recently Peirce (65) reported that the addition of urea to a ration containing a high proportion of potato starch increased wool production 32 per cent over that which resulted from the basal ration. The addition of starch to the ration of steers caused a substantial decrease in the digestion of total dry matter, but the addition of starch did not depress digestibility of dry matter when certain alfalfa hays were fed [Burroughs *et al.* (66)]. This was explained on the basis of an adequate or inadequate supply of nutrients for the growth of rumen microorganisms which were associated either with starch fermentation or roughage digestion. These investigators (67) observed that the digestion of corncobs by steers was increased by the addition of alfalfa leaf meal, either as a water extract or as ash. This effect was attributed to an unidentified factor in the alfalfa ash needed for the digestion of roughage dry matter. Swift *et al.* (68) found an increased digestibility of crude fiber, N. F. E.,[2] calories, and dry matter when alfalfa ash was fed as a supplement to sheep.

Using an artificial rumen technique, Burroughs *et al.* (64) investigated the effect of several feed supplements on cellulose digestion and found that dried distillers solubles, soybean-oil meal, and linseed-oil meal appeared to stimulate the microbial digestion of cellulose. These feeds were followed by cane molasses, corn, wheat, wheat bran, and cottonseed meal. Continuing their studies *in vitro*, Burroughs *et al.* (69) found that a complex salt mixture, the ash of alfalfa extract, autoclaved rumen liquid, or an autoclaved water extract of manure, added to the rumen inoculum, proved helpful to cellulose digestion.

Cellulose digestion and appetite in sheep were depressed by the administration of therapeutic doses of sulphanilamide [Oyaert *et al.* (70)]. The following antiseptics completely inhibited cellulose digestion *in vitro:* sodium arsenate, sodium cyanide, mercuric chloride, and iodoform [Meites *et al.* (71)]. Wasserman *et al.* (72) observed from studies, *in vitro*, that penicillin stimulated cellulose digestion at a low concentration, and neomycin stimulated at all concentrations used; but chloromycetin had an adverse effect. Crude fiber digestion in the intact steer was depressed by feeding aureomycin [Bell *et al.* (73)], whereas Chance *et al.* (74) showed that the ingestion daily of 0.5 gm. of crystalline aureomycin per steer stimulated the removal of crude fiber from the rumen.

Protein

It is universally recognized that legumes are usually higher in protein than grasses at the stage of maturity generally used by ruminants. Very immature legumes and grasses are high in protein on the dry basis, but decline with advance in maturity [Phillips *et al.* (44); Waite & Sastry (75); Sotola (49); Dijkstra & Brouwer (76); Ely (50); Armstrong *et al.* (46)]. The coefficient of digestibility of protein by ruminants also declines as the herbage matures [Sotola (49); Dijkstra & Brouwer (76); Burkitt (77); Swanson & Herman (78); Underwood & Moir (79); Crampton & Jackson (80); Daniel *et al.* (81); Colovos *et al.* (82); Dunlap & Coup (83); Richards & Reid (51); Ely (50)].

The crude protein of roughages contains from 10 to 15 per cent non-protein nitrogen which consists of free amino acids, nucleic acids, purine and pyrimidine bases, choline and betaine, alkaloids, peptides, urea, nitrates, etc. In the ruminant a part of the crude protein ingested is converted to ammonia by proteolytic enzymes produced by microbial cells, and the ammonia is synthesized by rumen microorganisms into microbial protein [McDonald (84)]. The ammonia not used immediately by bacteria is absorbed through the rumen wall and converted to urea by the liver. A part of the urea passes into the rumen again by way of the saliva, thus completing a nitrogen cycle; the rest is eliminated in the urine as urea. As was pointed out by Clark *et al.* (85), overdosing of urea into the rumen of sheep causes acute intoxication characterized by atony of the rumen, muscular spasms, and sudden death because of circulatory failure brought about by the rapid change of urea to ammonia. In the presence of sufficient readily available carbohydrate, an active rumen flora will utilize the ammonia rapidly as a source of nitrogen and so prevent its accumulation. McDonald (84) found that different protein sources were converted to ammonia very slowly, as compared with casein; about 40 per cent of the nitrogen of zein was converted to microbial protein. Cuthbertson & Chalmers (86) also showed that casein is deaminized rapidly in the rumen and is consequently of little value as a protein supplement when fed to sheep unless the rumen is bypassed and the casein is given directly into the duodenum. Chalmers & Synge (87) found that herring meal was superior to casein as a supplement for sheep, because of the slower production of ammonia. Moir & Williams (88) estimated that about 50 per cent of ingested nitrogen was converted to microbial protein in the fermentation vat of sheep.

Reed *et al.* (89) found that the digestibility and biological value of microbial protein were 63 and 79, compared to 101 and 80 for casein. McNaught *et al.* (90) found that dried rumen bacteria contained 44.4 per cent crude protein with digestibility and biological values of 73 and 88, respectively. Williams & Moir (91) studied the influence of feeding a constant level of different sources of nitrogen to growing lambs. The supplemental feed contributed 40 per cent of the total nitrogen. The mean biological values of the nitrogen of the different rations were: linseed meal, 80; subterranean clover

seed, 83; egg, 87; casein, 82; urea, 69; and urea plus methionine, 75. The mean total count of ruminal bacteria on the above rations were (millions per ml.): linseed meal, 24; subterranean clover seed, 25; egg, 41; casein, 43; urea, 18; and urea plus methionine, 44. It was concluded that different sources of nitrogen vary markedly in their biological value and in their capacity to promote bacterial growth in the rumen of lambs and mature sheep. These results are in agreement with the findings of Loosli & Harris (92) and Lofgreen *et al.* (93) with respect to urea plus methionine, both on the basis of nitrogen retention and biological values. The biological values of nitrogen for lambs were found to be: whole egg, 80; linseed meal, 76; urea plus methionine, 74; and urea, 71.

Loosli *et al.* (94), using intact sheep and goats, were the first to show the synthesis by microorganisms in the rumen of sheep of the 10 amino acids essential for rat growth; a ration of cellophane, corn starch, glucose, lard, minerals, vitamins A and D, and urea as the only significant source of nitrogen was provided. Duncan *et al.* (95) fed essentially the same ration to fistulated steers and confirmed the synthesis of the 10 essential amino acids. The entire rumen contents were removed before feeding and weighed, sampled, and returned and then repeated six hours later. When the amino acid composition of the rumen microbial protein was expressed as a percentage of the sample, no consistent difference was observed between the values obtained before feeding and six hours after, but the total amounts in the rumen indicated extensive synthesis. The amino acid pattern of the mixed proteins in the ingesta of the steers on the purified ration was fundamentally similar to that found in a steer on a natural ration. Black *et al.* (96) concluded that rumen microorganisms furnish the essential amino acids, and the tissues of the cow synthesize the nonessential amino acids.

The value of urea as a source of nitrogen for ruminants has been reviewed by McNaught & Smith (97). Using an artificial rumen technique, Burroughs *et al.* (98) investigated the utilization of urea in the presence of purified proteins and protein-rich feeds and concluded that the nitrogenous requirements of rumen microorganisms are relatively simple in nature, essentially involving only ammonia and not involving the more complex forms of nitrogen, i.e., amino acids. Arias *et al.* (99) found that dextrose, cane molasses, sucrose, starch, cellulose, or ground corncobs, added to the fermentation flasks as sources of energy, aided in the utilization of urea and in cellulose digestion. Burroughs *et al.* (100) reported that phosphorus and iron were effective in stimulating urea utilization and cellulose digestion by rumen microorganisms. Their data also indicated that an unidentified factor was needed for urea utilization and cellulose digestion.

Hamilton *et al.* (101) using nitrogen balance as a criterion, confirmed their previous findings that urea is as satisfactory a source of nitrogen for growing lambs as that from most ordinary feeds, provided at least 25 per cent of the feed nitrogen is in the form of preformed protein and provided further that the total protein equivalent of the ration does not exceed 12

per cent. Watson (102) demonstrated the formation by ruminants of body protein from urea labeled with N^{15}. Urea added as a supplement to a low-protein diet, high in crude fiber and low in starch, failed to increase wool production, according to Peirce (103), but the substitution of an equivalent amount of nitrogen in the form of wheat gluten increased wool production 35 per cent.

There is no doubt that simple nitrogenous compounds, such as urea, are used by rumen microorganisms to synthesize protein for the use of the host; most workers have found that such compounds are not as effective as the nitrogen from natural proteins. This may be due to a too rapid conversion to ammonia, in which case the excess ammonia would be absorbed through the rumen wall and would not be available to the rumen bacteria at the peak in population (two to four hours). The conventional digestibility studies of proteins do not give reliable information as to whether absorbed nitrogen has entered the animal as ammonia or as essential amino acids [Synge (104)]. Protein requirements of ruminants used in practice should take this into consideration.

The microbial synthesis of methionine from inorganic sulfur has been demonstrated by Loosli *et al.* (94) and Duncan *et al.* (95). Block & Stekol (105) described the rapid conversion in the cow of orally ingested S^{35} (as sodium sulfate) into cystine and methionine of milk proteins. Block *et al.* (106) reported that milk proteins showed considerable activity within three hours after the oral administration of radioactive sodium sulfate (S^{35}) to a goat. Cystine and methionine were synthesized in the rumen at approximately equal rates. This is in agreement with the observation of Johanson *et al.* (107). According to Gallup *et al.* (108), rumen microorganisms are able to synthesize sufficient methionine from inorganic sulfur to meet the needs of the growing lamb. These investigators also reviewed the literature on this subject. The sulfur content of feeds consumed by ruminants show wide variations (37). Most of the roughages, however, appear adequate in this element.

Burroughs & Gerlaugh (109) and Burroughs *et al.* (110) have shown that the supplementation of low-protein rations with protein concentrates increases the digestibility of the dry matter in the roughage. Rumen bacteriological studies, with and without the addition of casein, revealed differences which correlated closely with the degree of roughage digestion. The minimum protein requirement of cattle is suggested by Burroughs *et al.* (111) as representing a protein (nitrogen) requirement for the growth of the rumen microorganisms directly involved in roughage digestion. Gallup & Briggs (112) increased the dry matter digestion of prairie hay in steers by means of cottonseed meal supplementation. Moir (113) attributed the seasonal variation in ruminal microorganisms of grazing sheep to seasonal changes in the protein content of the herbage.

The most economical sources of protein for ruminants are legume forage and immature grasses.

Fat

The literature pertaining to the chemical composition of crude fat in roughage has been reviewed by Sullivan & Garber (114). Herbage is low in ether extractable substances and only about 32 per cent of this fraction is true fat. Many years of research on the fat requirements of cows for milk production were summarized recently by Loosli *et al.* (115). Increased milk production appeared to follow increased fat percentage in the grain supplement up to about 4 per cent. Leroy & Bonnet (116) found that butterfat and milk production of cows declined when less than 3 per cent fat was included in the ration on the dry basis. Other workers were unable to substantiate the contention of Loosli *et al.* and Leroy & Bonnet [Monroe & Krauss (117); Huffman & Duncan (118)].

The literature dealing with the effect of different feed supplements on the percentage of fat in milk has been reviewed by Moore *et al.* (119). It is difficult to increase the percentage of fat in milk by feeding, except for short periods of time. Many investigators have reported marked reductions in the percentage of butterfat in milk by feeding oils high in unsaturated fatty acids. As pointed out by Moore *et al.*, this phenomenon can be prevented by feeding the usual amount of cod liver oil every two hours rather than twice daily. The fat-depressing effect of such oils appears to be associated with the "flooding" of the digestive tract. According to Breirem (120), the cows in Norway produced milk abnormally low in fat during World War II. This was attributed to under feeding of energy and protein and the feeding of marine oils.

The feeding of small amounts of roughage or finely ground roughage resulted in a marked decline in the percentage of fat in milk with no significant effect on milk yield [Powell (121)]. These results were confirmed by Loosli *et al.* (122), but not by Espe & Cannon (123). The cows used by Espe & Cannon were fed long hay or a liberal amount of corn silage which probably explains the failure to confirm the work of Powell. The effect of three levels of hay feeding on butterfat and milk production of dairy cows was studied by Balch *et al.* (124) who reported that with six pounds of hay per cow daily, fat percentage dropped, accompanied by an increase in milk yield. The cows fed two pounds of hay per day declined in milk yield and in the fat content of the milk. The work of McClymont (125) suggests some deficiency in the roughages for the fat content of milk declined when the cows were turned to young oat pasture.

Indirect evidence of the relationship of rumen microorganisms to butterfat and milk production has received some consideration. Stoddard *et al.* (126) reported that cows fed six pounds or less of hay daily with concentrates, *ad libitum*, showed a marked decrease in butterfat percentage, which returned to normal on the oral administration of acetic acid. The administration of propionic acid had no effect. It was found by Tyznik & Allen (127) that the depression of butterfat percentage by a low roughage

ration was associated with increased production of propionic acid and a decreased production of acetic acid. No change occurred in butyric acid production. The fat percentage increased within 24 hours following the first feeding of sodium acetate and reached an approximately normal level when the cows were eating 454 gm. daily. Zelter (128) showed that when cattle were fed acetate and butyrate, or silage rich in these acids, increased milk and butter fat production resulted.

The principal short chain fatty acids produced during microbial fermentation in the rumen are acetic, propionic, and butyric acids. The energy value of the acids was pointed out by Elsden & Phillipson (14).

Early experiments, reviewed by Elsden & Phillipson (14), indicated that acetic acid was produced in greater amounts than either propionic or butyric acids in the rumen of sheep. Similar results were reported by Crane & Hansen (9) in the goat and by McClymont (129) in the cow. Using an *in vitro* method of studying the proportions of fatty acids produced during fermentation of rumen contents from sheep, Gray & Pilgrim (130) showed that the proportion of propionic acid exceeded acetic acid during early stages, but later acetic acid exceeded propionic acid. These investigators (131) also reported that fermentation of both cellulose and hemicellulose gave rise to approximately equal parts of acetic and propionic acids with a very small proportion of butyric acid. The presence of protein, however, led to the formation of an increased proportion of butyric acid.

In recent experiments Kiddle *et al.* (132) and Masson & Phillipson (133) showed that acetic acid forms the largest part of the mixture of acids absorbed from the rumen in grass-fed sheep and that it is probable that the preponderance of acetic acid in the rumen is due to its greater production rather than to preferential absorption of the remaining acids. The concentration of butyrate in the blood leaving the rumen was less than that of acetate or propionate when equimolar solutions of these salts were present in the rumen which suggested the possibility of butyrate metabolism in the rumen wall.

The relative proportions of volatile fatty acids produced during microbial fermentation may depend upon the nature of the rations fed. In a limited experiment with lambs, Phillipson (134) reported that on a heavy corn ration the proportion of total volatile acids, such as propionic, was not the usual 20 per cent, but about twice this amount. Johns (135) reported that the ratio of acetic to propionic acid in the rumen was influenced by the carbon dioxide tension of the medium.

Fundamental work by Pennington (136), which may apply to the ketosis problem, showed that ketone bodies were produced from butyric and to a lesser extent from acetic and propionic acid when rumen epithelium was used *in vitro*. The utilization of propionic acid was more rapid under an atmosphere of carbon dioxide than under an atmosphere of oxygen. Ketone bodies were produced by the epithelium of the reticulum and omasum as well as

by liver tissue, but not by the epithelium of the abomasum, cecum, and kidney. The epithelium of the forestomachs of sheep was about as active, per unit of weight, as liver tissue in the production of ketone bodies.

Branched chain lower fatty acids were found in the rumen of sheep on a variety of rations by El-Shazly (137, 138). A significant proportion of the C_4 acids was isobutyric acid, and branched chain isomers often made up the greater part of the valeric acids. Increased ammonia production was correlated with increased concentrations of isobutyric and valeric acids, which are considered to arise from microbial attack on protein in the rumen. Considerable evidence has accumulated which indicates that these acids may be ketogenic, especially isovaleric acid. The author postulated that these branched chain fatty acids may play a part in some of those forms of ketosis in ruminants that have been attributed to high-protein rations.

The oral administration of acetic, propionic, lauric, oleic, lactic, or succinic acid to goats caused no significant rise in blood ketones, according to Schultz & Smith (139), but the administration of butyric, caproic, caprylic, and capric acid caused a 5 to 10 per cent increase in blood ketones. According to Miller & Allen (140), the oral administration of from 100 to 450 gm. of sodium acetate daily to 23 cows with clinical symptoms of ketosis resulted in 12 recoveries without other treatment. The treatment of 19 cows with ketosis by the oral administration of sodium propionate at levels of 56 to 225 gm. daily resulted in recovery of all cows, according to Schultz (141); the proportions of acetic, propionic, and butyric acids were normal. Isopropyl alcohol was found in the blood, milk, and rumen contents of cows suffering from ketosis, whereas there appeared to be none present in normal animals [Robertson *et al.* (142)].

Other investigators have attributed the occurrence of ketosis in cattle to the type of ration fed [Hoflund & Hedstrom (143); Hvidsten & Homb (144); Breirem *et al.* (145)]. The prevention of ketosis (pregnancy disease) in ewes is brought about by liberal feeding during the last two months of pregnancy, according to Sampson (146).

Minerals

Reviews concerning the role of copper in nutrition have been prepared by Marston (147) and McElroy & Glass (148). Fluorine in ruminant nutrition was adequately reviewed by Mitchell & Edman (149). A few of the more recent developments in calcium, phosphorus, and cobalt nutrition will be reviewed.

Calcium.—As a rule, roughages are good sources of calcium which probably accounts for the uncommon occurrence of calcium deficiency among ruminants under natural conditions. By using radioactive calcium (Ca^{45}), Hansard *et al.* (150) found that circulating calcium is secreted into all parts of the digestive tract of the bovine, with the small intestines playing the major role. About 19 per cent of the fecal calcium was derived from sources other than the feed, presumably from body stores. They interpreted their

results to mean that calcium in the bones is not completely exchangeable with the calcium in the blood plasma. The calcium ions in the soft tissue appear to be interchangeable with those of the plasma.

In studies with lactating goats, Visek *et al.* (151) found that the amount of calcium absorbed from the gastrointestinal tract and appearing in the blood was closely related to the calcium requirements of lactation. The higher the level of milk production, the greater was the percentage of calcium absorbed.

Franklin *et al.* (152) produced severe hypocalcemia in sheep by feeding low-calcium, high-phosphorus rations. Blood serum values as low as 2.7 mg. per cent were observed. The hypocalcemia could be prevented or cured by supplementing the ration with either legume roughage or finely ground limestone, but not by bone meal. These results are difficult to explain in view of data reported in the literature.

Ward *et al.* (153) found that dairy cows which developed parturient paresis were in severe negative calcium balance just prior to parturition, while the normally calving cows were in positive calcium balance during this period. All cows were in negative calcium balance following parturition. According to Hibbs & Pounden (154), the administration of 5 to 30 million I.U. of vitamin D daily for one month prior to parturition may have prevented parturient paresis in dairy cows.

Reviews covering the role of calcium in the nutrition of ruminants include: cattle and goats by Visek *et al.* (151), and sheep by Franklin *et al.* (152).

Phosphorus.—When Shirley *et al.* (155) fed a grass grown on soil which had been fertilized with radioactive phosphorus (P^{32}), wide distribution of the isotope was found in the animal tissues, which indicated that the plant not only utilized the applied fertilizer, but also contained it in such a form that it was readily assimilated by the young animal. The use of radioactive phosphorus enabled Kleiber *et al.* (156) to determine the exogenous and endogenous fecal phosphorus in two lactating cows fed at different levels of dry matter intake. They concluded that the secretion rate of endogenous phosphorus was correlated positively with the level of feed intake. Endogenous fecal phosphorus was used to calculate the "true" digestibility of phosphorus. The cow on the highest feed intake showed an apparent digestibility of 12 per cent, while the "true" phosphorus digestibility was 50 per cent. The cow on the lowest feed intake excreted even more phosphorus in the feces than was consumed in the feed. The "true" phosphorus digestibility, however, amounted to 64 per cent.

According to Chandra & Owen (157), the administration of thyroxine to milking cows resulted in an apparent negative digestibility of calcium, associated with an increased positive phosphorus balance. This unusual retention of phosphorus reflects the effect of thyroxine in increasing the proportion of phosphorus bound in the various tissues of the body in ester form by increasing the phosphorylating enzymes in the tissues.

The mean inorganic phosphorus content of the blood plasma of cattle pastured on semidesert range forage was 3.53 mg. per cent [Watkins & Knox (158)]. These analyses, together with the calculated phosphorus intake, indicate that the cattle received considerably less phosphorus than recommended by the widely used standards. The inorganic phosphorus values of the blood plasma were definitely lower than is considered by other investigators to be the critical level.

The effect of feeding steers an excess of calcium with borderline and phosphorus-deficient rations was investigated by Lewis *et al.* (159). The feed consumed per 100 kg. of body weight and the rate of gain were reduced by borderline or deficient phosphorus intakes. Excess calcium added to a borderline phosphorus ration further reduced feed consumption and the rate of gain. When excess calcium was added to a phosphorus-deficient ration, feed consumption decreased, but the rate of gain was not affected. The symptoms of phosphorus deficiency in order of their appearance were: impaired feed utilization, reduced rate of gain, poor feed consumption, depraved appetite, emaciation, rough hair coat, stiffness in the hind legs, enlargement of the knees and hocks, and alteration of body conformation.

Davis (160) reported that a borderline copper deficiency is characterized by a failure to utilize phosphorus adequately and results in symptoms resembling phosphorus deficiency. The symptoms are alleviated by copper but not by phosphorus supplementation.

Gallup & Briggs (161) used lambs weighing from 25 to 31.8 kg. to study the minimum phosphorus requirement with and without urea. The 10 rations varied from 0.13 to 0.30 per cent phosphorus and from 0.14 to 0.84 per cent calcium. Lambs were in phosphorus equilibrium when phosphorus intakes varied from 4.2 to 4.6 gm. per 100 kg. of body weight. Amounts below this level resulted in negative phosphorus balances, and amounts above this level resulted in positive phosphorus balances. The calcium requirement appeared to be slightly greater than the phosphorus requirement.

Cobalt.—Beeson (162) and Marston (147) recently reviewed the literature pertaining to the role of cobalt in ruminant nutrition. Cobalt deficiency is the most prevalent mineral deficiency among ruminants. In many instances a deficiency of this element has been attributed to phosphorus deficiency since the lack of appetite, depraved appetite, and emaciation are symptoms common to both.

The fact that ruminants appear to require elemental cobalt, whereas nonruminants do not respond to inorganic sources of this elment, has stimulated research on this problem in many regions of the world. According to Marston & Lee (163) and Smith *et al.* (164), cobalt-deficient sheep do not respond to injected cobalt, but Ray *et al.* (165) reported a very slow recovery.

The administration of 0.1 mg. of cobalt daily, either intravenously or in the duodenum, did not cure cobalt-deficient lambs, but larger quantities of cobalt allowed growth to occur at a rate similar to that of lambs receiving 0.1 mg. of cobalt daily by mouth [Phillipson & Mitchell (166)]. The rate of

response of sheep to injected radioactive cobalt was studied by Keener *et al.* (167). Their results indicate that a very small amount of cobalt passes from the blood stream into the rumen contents, either directly through the saliva or by means of regurgitation from the small intestine.

Gall *et al.* (168) showed that cobalt supplementation increased rumen bacterial counts of sheep, whereas a cobalt deficiency caused marked alterations in the types of bacteria. The bacteriological changes were not due to a lower feed intake.

The discovery of cobalamin (vitamin B_{12}) brought about a new approach as to how cobalt is utilized in the ruminant. Several investigators have shown that the feeding of cow manure to nonruminants such as the chick, stimulated growth. The active principle was later shown to be cobalamin. Hale *et al.* (169) used chicks as assay animals to determine cobalamin and found that rumen ingesta from cobalt-supplemented sheep produced greater chick growth than that from cobalt-deficient sheep.

The supplementation of cobalt-deficient sheep with crystalline cobalamin had no apparent effect [Becker *et al.* (170)]. Subcutaneous injection of 10 mg. of pteroylglutamic acid per lamb daily or the simultaneous injection of 1 mg. of cobalamin and 1 mg. of cobalt per lamb daily were ineffective in cobalt-deficient lambs. The subcutaneous injection of 15 units of a purified liver extract (U.S.P.) daily per lamb was quite effective in the treatment of cobalt deficiency. The oral administration of the same dosage level was of no value. Smith *et al.* (171) found that when 0.15 mg. or more of cobalamin were injected, the cobalt-deficient lambs responded favorably in appetite, body weight, and hemoglobin level. It was concluded that cobalamin is an important intermediary in cobalt metabolism of sheep. Koch & Smith (172) used three groups of cobalt-deficient lambs: one group received cobalt orally; a second group received injected cobalamin; and a third group received injected hydroxo-cobalamin (vitamin B_{12b}). All three groups made equal gains in weight. These results confirmed the findings of Smith *et al.* (171) that injected cobalamin will alleviate the symptoms of cobalt deficiency in lambs and, furthermore, that hydroxo-cobalamin is as effective in this respect as cobalamin. The amount of cobalt in this vitamin is so small that the activity cannot be due to cobalt per se. Other functions if any, of cobalt in ruminant nutrition remain to be elucidated.

Measuring Nutritive Value of Rations

In the assessment of the nutritive value of rations for ruminants, consideration must be given to the nutrient requirements of rumen microorganisms as well as of those of the host. The nutritive value of roughage depends on the digestibility, appetite (which depends on the rate of rumen digestion of celluloses and hemicelluloses), and the balance of nutrients made available to the tissues of the animal. Some of the problems involved in assessing the productive value of rations consisting of forage alone and with various concentrate supplements were pointed out by Kriss (173) who

showed that the conversion of total digestible nutrients (T.D.N.) to calculated net-energy and starch equivalent is a doubtful procedure. Raymond (174) outlined the problem of measuring the nutritive value of forage crops and emphasized the fact that the starch equivalent method of feed evaluation lacks experimental evidence and does not merit the accuracy with which it is often credited. The work of Burroughs *et al.* (175) showed that corncobs fed with good alfalfa hay and grain were almost as productive per unit of T.D.N.[2] for body gain in steers as corn grain. Huffman *et al.* (176, 177) reported an increased production of milk when a portion of hay in an all-hay ration was replaced with grain and either wheat straw or peanut hulls. The increase in milk production was associated with decreased intakes of starch equivalent. Some immature hays were superior to more mature hays for milk production when fed on an equal starch equivalent basis. The increased milk production was explained by the presence, in grain and immature hay, of unidentified lactation factors which were deficient in the more mature hays. Starch equivalent values of hays do not evaluate them adequately for milking cows. Burroughs *et al.* (175) and Huffman *et al.* (176) support the modern concept of a balanced ration, as first proposed by Forbes (178), and tend to support the contention of Mitchell (179) that the net availability of the metabolizable energy of all balanced rations is the same. The high productive power of young grass, reported by Levy (180) for New Zealand pastures, also suggests the desirability of using a balanced energy system of feed evaluation which postulates a flexible "true" net energy which goes up with improved balance of the ration. Bartlett (181) attributed the high milk-producing power of young grass to the presence of estrogen-like compounds.

According to Raymond, T.D.N.[2] is the most practical basis for feed evaluation due to the economy and ease of determination. A more accurate method, however, of determining digestible energy is by the use of the bomb calorimeter, according to Raymond (174) and Lofgreen (182). Several species of forage crops compared reasonably well on the basis of metabolizable energy, digestible energy, digestible dry matter, and T.D.N., according to Swift *et al.* (183). These measurements of digestible energy, however, do not take into consideration the balance of the ration. It is imperative to improve the balance according to present day knowledge in order for the animal to convert the digestible energy efficiently to milk, meat, and wool. This is the method used in the nutrition of nonruminants, such as pigs and chickens.

Many studies dealing with methods of improving the accuracy and economy of determining digestible nutrients have been made. Schneider & Lucas (184) concluded that accurate average digestibility data can only be obtained when a feed is studied by a relatively large number of co-operators, each investigating several samples of the feed.

In addition to conventional digestion trial data, various reference substances have been used to determine digestibility coefficients by the ratio

technique. Kane *et al.* (185) reported that the use of indigestible materials such as lignin and chromium oxide shows promise of saving time, labor, and the expense involved in the present cumbersome method of conducting digestion trials. They reviewed the literature on this subject.

A reliable determination of the digestibility of pasture, as grazed, has long been needed. The possibility of using a naturally-occurring plant chromogen has been investigated by Reid *et al.* (186). The possibility that the methoxyl content of forages or feces or both may prove to be equal or superior to the lignin content, as an index of the digestibility of forages, was suggested by Richards & Reid (51). The determination of methoxyl is simpler than the determination of lignin. In addition, methoxyl is a distinct chemical radical and this is not true of lignin. The literature pertaining to the use of fecal nitrogen as a measure of dry matter intake and of digestibility of organic matter in forage was reviewed by Homb & Brierem (187).

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PHOTOSYNTHESIS[1,2,3]

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Introduction

Within the last two years there have appeared upwards of a dozen review articles or monographs devoted entirely or in large part to considerations of research progress in photosynthesis (1 to 12, 78). The most ambitious of these is the long awaited Volume II of *Photosynthesis and Related Processes* by Rabinowitch. This monograph now is effectively divided into three volumes, having grown so much by postwar accretion that Volume II had to be published in two parts. Whereas Rabinowitch's first volume (44) dealt mostly with the chemistry of photosynthesis, the second volume is devoted primarily to the physico-chemical aspects of the subject: to spectroscopy and the fluorescence of chlorophyll and other pigments, and to the kinetics of the photosynthetic process. This most competent presentation and analysis of numerous heterogeneous contributions is a reference work of forbidding length. Nevertheless it achieves a remarkable readability. For those who require a general and balanced but much more condensed treatment of the whole subject, Whittingham's review (4) is recommended.

In view of the number and detailed nature of relatively recent reviews on several aspects of photosynthesis, it seems justifiable to direct the present discussion chiefly toward the nonspecialist who may appreciate a general treatment of some of the major problems from a new or at least different viewpoint. Since it is necessary to comply, however painfully, with strict space limitations, a review restricted to a few topics should be more useful to the general reader and certainly more interesting to prepare, than a comprehensive tabulation of all recent contributions presenting only a vestigial discussion of any of them. This review will discuss five topics which coincide with certain interests of the authors. These topics are: (*a*) The efficiency of photosynthesis (minimal quantum requirement); (*b*) The biochemical pathway of carbon during photosynthetic assimilation; (*c*) Enzymes in some direct way implicated in the photosynthetic process; (*d*) Photosynthesis and phosphorus metabolism; (*e*) The source of photosynthetic oxygen.

The Quantum Requirement of Photosynthesis

Even assuming perfect efficiency, from simple energetic considerations, the quantum requirement of photosynthesis cannot be less than about three

[1] The survey of the literature pertaining to this review was concluded in December, 1952.

[2] The following abbreviations will be used: DNP, 2,4-dinitrophenol; ATP, adenosinetriphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced TPN; HCN, hydrogen cyanide.

[3] Reprinted with permission of the authors from the *Annual Review of Plant Physiology*, Volume 4, June, 1953.

although Franck (13) has presented cogent arguments against the possibility of the photosynthetic efficiency approaching 100 per cent.

Partly by reason of reaction stoichiometry, various theories of mechanism have suggested a minimal photosynthetic quantum requirement of either exactly four or exactly eight, as seemed in keeping with experimental determinations accepted at the time. Whether one thinks of carbon dioxide reduction in terms of a unique photochemical reduction occurring in close association with the chlorophyll or, as some biochemists now do, in terms of a purely thermochemical reaction sequence driven by quite nonspecific reductants chemically and physically remote from the pigment system, there is broad agreement that the photochemical parts of ordinary photosynthesis, photoreduction, or the Hill reaction, have in common the photolysis of water and the generation of an oxygen precursor whose nature does not depend upon the ultimate oxidant. Accurate measurements on the quantum requirements of several of the "aberrant" photosyntheses are now available (14, 15, 16). Understandably, the requirements were found to be essentially the same regardless of the large differences in energetic efficiency which this involves.

The experimental determination of the minimal quantum requirement of *Chlorella* photosynthesis has become one of the most strenuously contested problems in all of biology. The history of this controversy goes back to the earliest determinations when a quantum requirement of about four (energetic efficiency above 70 per cent) was first computed by Warburg & Negelein (17, 18) on the basis of manometric studies. Working in acid medium they employed the "two vessel" method to measure simultaneous oxygen and carbon dioxide pressure changes. The basic assumption was made that light had no effect on the respiratory rate and light readings were corrected by the rate of respiration in the dark. Very dense *Chlorella* suspensions were used, thereby simplifying the photometry since all incident light was assumed to be absorbed by the suspension. Subsequent work by Warburg and collaborators has shown that the high efficiencies they reported were not dependent on any of the above special conditions but were perhaps the result of certain special combinations of experimental conditions.

A group of workers at the University of Wisconsin was unable to confirm Warburg's initial result by several independent methods (19, 20, 21). Rieke (22), and Emerson & Lewis (23) found that Warburg's results were indeed reproducible if the latter's methods of measurement and computation were exactly followed, particularly with regard to the "sequence and duration" of illumination. If light and dark periods were of greater duration or if computations were based on different parts of the respective intervals, lower efficiencies invariably were obtained, a circumstance suggestive of some systematic error in the particular method leading to the calculation of exceptionally high efficiency. Emerson & Lewis (23, 24) elucidated certain transitory phenomena which occurred at the beginning and end of an illumination, and which caused the quotient of gas exchange to deviate

temporarily and considerably from unity. This effect, combined with a somewhat different equilibration lag in the two vessels of different gas-liquid volume ratios, apparently was sufficient to account for systematic errors of several hundred per cent, as well as for a marked dependence of computed efficiency upon the particular schedule of manometer readings and times of light exposure. There then seemed little doubt that the technically elegant work of Emerson & Lewis had explained the discrepancy between the findings of Warburg & Negelein and the results of other laboratories. However, Warburg rejected the explanation based upon errors in his experimental design or interpretation and, returning to the problem but largely discounting criticisms of his earlier work, he repeated those measurements with minor modifications in technique and obtained the same end result (25). There soon appeared a flood of contributions, none of which confirmed the low requirement of about four quanta but, in general, ranged upward from a value of about eight or nine (15, 26, 27, 28). The argument has swung back and forth between Warburg and co-workers on the one side (29, 30, 31, 32, 33), and Emerson and co-workers on the other side (27, 34). An attempt by Emerson and Warburg to arrange collaborative experiments proved fruitless. By the latest experiments, the breach has only been widened as Warburg's laboratory reports a quantum requirement as low as about 2.8 for *Chlorella* photosynthesis under special experimental conditions (35).

The underlying disagreement relates to methodology. Like all quantitative procedures, the manometric method has definite limitations and the quantum efficiency problem requires that the method be used under conditions where the magnitude of systematic error becomes uncertain, debatable, and possibly very large. It is not surprising that opposing schools should find grounds for disagreement in each other's experimental designs. While it may be unduly pessimistic to suggest that the argument is at an impasse, it now seems most unlikely that anything approaching agreement will be achieved by the protagonists of these two schools, in as much as the profound difference in their positions rests upon subtle points of experimental design and interpretation.

The present reviewers suggest that the versatile, two vessel, manometric method is neither adequately sensitive nor capable of sufficiently unequivocal results to obviate various arguments over the appropriate interpretations of these results. Specifically, disagreement exists over the quotient, $\Delta CO_2/\Delta O_2$, in light and in darkness, over reported anomalies in the time course of gas exchange during the first moments after transition from dark to light or the reverse (and corresponding transitory fluctuations in the quotient), and over the significance of different time lags in the gaseous equilibration between phases in the two vessels. Since the basic controversy thus concerns the method and the instrumentation itself, a persistent loyalty to manometric methods now seems contraindicated. To provide decisive information, methods based on other physical or chemical principles could be resorted to advantageously. The usefulness of the more elaborate

physical methods generally stands in inverse ratio to their complexity and it is understandable why none has achieved the popularity of the simple and versatile manometric or volumetric procedures. Nevertheless, it seems particularly desirable in this case to bring different methods to bear upon the problem. There are special physical methods with excellent specificity and very small inertia. Uncertainty over the composition of the gases responsible for pressure changes observed with a manometer (i.e., fluctuations of the quotient) can be eliminated by unambiguous assay methods such as infra-red spectrometry (36), electrical impedance measurements (37), and thermal conductivity measurements [(38, 39) for carbon dioxide], or measurements of magnetic susceptibility (40, 41) and polarography [(20) for oxygen], or mass spectrometry [(42) for both gases].

What is desired most is an analytical method specific for oxygen and showing no significant time lags in response to changes in oxygen tension. Time lags for instrumental response alone can be made negligible in each of the above methods but the major lag, that of gaseous equilibration across the interface between the suspension and the gas phase, is abolished only by the elimination of the gas phase. This can be done with the oxygen electrode. Therefore, among the most convincing determinations of the photosynthetic efficiency of *Chlorella* suspensions are those made with the dropping mercury electrode (28) and, more recently, those in which the platinum electrode was employed (43). Values of the minimal quantum requirement for *Chlorella* photosynthesis determined by these methods were in the range of about six to ten. While it is, of course, axiomatic that only the minimal values (within the range of experimental error) are of interest for the solution of this problem, it is equally true that a valid result should be confirmable by independent methods of measurement and the burden of proof clearly rests with those who claim the lowest quantum requirement.

For those not intimately concerned with the problem of minimal quantum requirement, the literature of this controversy has reached a forbidding level of technical complication and of concern with experimental detail.

Preoccupation with the polemic between Emerson and Warburg does not justify losing sight of the underlying assumptions which are made by those who attempt absolute photosynthetic rate determinations. One assumption made, or at least implied, is that respiration is a function which proceeds concomitantly with assimilation at the same rate as in the dark. Measurements of the photosynthetic rate customarily are corrected for respiration as measured in the dark and, since light measurements are carried out at intensities near or even below compensation, this correction is large. The use of the dark control, involving the often tacit assumption that light is without effect on the respiratory rate, has been a generally accepted device and its validity usually has not been specificially questioned although, as Rabinowitch points out, the possibility of a photoeffect on respiration "is a nightmare oppressing all who are concerned with the exact measurement of photosynthesis" (44, p. 569).

Warburg *et al.* (45), ignoring previous literature dealing with the question

of a light effect on respiration, reported that oxygen uptake by a dense *Chlorella* suspension was unaffected by red light of low intensity when the suspension was severely depleted of CO_2 by the use of NaOH in the side arms of the reaction vessel. According to the data given, gas uptake was, in fact, reduced by 2.5 per cent in the light. This small effect, apparently within experimental error, was ignored. However, since only an estimated 5 per cent of the cells were exposed to the light beam due to prevailing conditions of illumination and suspension density, it is doubtful if the result was as significant as the authors claimed. From this single type of experiment on but one algal strain, sweeping conclusions appear unjustified. Nevertheless these authors believed their result demonstrated a general lack of dependence of the respiration of green cells upon illumination. Actually, the more reasonable interpretation of their data taken at face value is that it demonstrated just the opposite. While the low CO_2 tension prevailing in their experiments severely inhibited photosynthesis, the suggestion that inhibition was complete is implausible as was pointed out by Whittingham (4), Rabinowitch (1), and by Kok (46) who also studied the matter experimentally. Kok (46), using a technically superior manometric method, could not reproduce Warburg's result. Not only was he unable to suppress photosynthesis completely by absorbing CO_2 efficiently, but he also observed induction effects consisting of enhanced oxygen utilization in the light. If there remained any residual photosynthesis under Warburg's experimental conditions then the observed lack of an effect of light must have been the result of respiratory stimulation. The manometric technique is, in principle, incapable of determining the true intensity of respiration if the photosynthetic rate is in doubt. The present reviewers find the circular reasoning of Warburg *et al.* unconvincing.

There is an extensive literature on the effects of light on respiration most recently reviewed by Weintraub (47), Rabinowitch (44), and Whittingham (4), but, until a few years ago, there was no convincing evidence that red or yellow light, as employed in critical quantum requirement studies, influenced *Chlorella* respiration in the steady state. However Rieke (22), Kok (46, 48, 49) and Van der Veen (50) have published data showing a nonlinear relation between light intensity and rate of oxygen exchange in the region of compensation. The data are fitted by two straight lines intersecting near or somewhat above compensation. Also, light curves measured by Burk and by Warburg are interpreted by these authors as curvelinear but they could as easily be interpreted as Kok does his curves, viz., as intersecting straight lines. Quantum requirements for photosynthesis calculated for data obtained below compensation often were in the neighborhood of four while above compensation the slope of the light curve indicated that the requirement was about twice this value. The "Kok effect" as it has come to be called, is readily interpreted as a photoinhibition of respiration which is proportional to light intensity and reaches its maximum approximately at compensation.

It seems that the effect is not as highly reproducible as might be desired

and its reality is not yet universally accepted. Also, the most convincing evidence in its favor applies to experiments in a medium of pH 7 or higher so that its application to most of the critical quantum requirement studies in other laboratories is uncertain. Emerson (1, p. 1118) has not observed the Kok effect, for his measurements of *Chlorella* gas exchange were almost linear with light intensity. However, Warburg (25, 51) and others report more curvilinear relations with points sufficiently scattered to prevent differentiating with certainty between a smooth curve and one of intersecting linear segments as suggested by Kok.

In an attempt to reconcile conflicting quantum requirement claims, Franck (52) revived the concept of photoreversal of respiratory reactions. He proposed that, under certain conditions affecting chloroplast permeability, the photoreduction of respiratory intermediates takes precedence over CO_2 assimilation. This process, energetically feasible in Franck's scheme with about half the quantum requirement of photosynthesis, was visualized as accounting for the Kok effect and for the exceptionally high calculated efficiencies of photosynthesis under Warburg's special conditions. At the time of its publication, Franck's proposal admittedly was not entirely consistent with all the known experimental results which apparently were germane. Later his proposal was modified and amplified (13), taking all data into consideration. There is not space here to develop Franck's arguments in favor of a photoreversal of catabolism under special conditions as an explanation of the quantum requirement anomaly.

Recently a direct approach was made to the problem of a photoeffect on *Chlorella* respiration. Brown (53) used tracer oxygen in the gas phase to tag the respiratory gas uptake of the algal suspension. Photosynthetic oxygen was untagged. Accordingly, the rate of tracer oxygen uptake, followed with a mass spectrometer (42), was a measure of the respiratory rate regardless of illumination of the cells. In slightly acid medium and at various intensities of white and of red light from intensities below up to several times compensation, there was evidence of neither photoinhibition nor photoenhancement of the respiration of several strains of *Chlorella*. Therefore, at least under the conditions used in these studies (which purposely were made comparable to those of published quantum requirement determinations), the use of a dark control to correct for respiration during the light exposure was found to be valid. Of course, there remains the possibility that circumstances requisite to demonstrating photoinhibition of respiration were not obtained.

A recent development in the field of quantum requirement measurements was contributed by Burk, Warburg, and various co-workers and concerns the reported kinetic separation of photochemical oxygen production from a nonphotochemical but light-augmented "combustion" process (35, 54 to 59). Using familiar manometric principles they measured *Chlorella* gas exchange in equal and alternating light and dark intervals of short duration. With periods as short as one or two minutes, their results were interpreted

as showing a rate of oxygen production in the light far greater than any previously encountered. In the dark, a combustion process (presumably not identical with ordinary respiration) proceeded at an accelerated rate, in extreme cases at tenfold the normal respiratory rate. It was proposed that for an integrated series of dark and light intervals (or for low intensity continuous light) the minimal quantum requirement is about three. However, for the photochemical partial reaction, the quantum requirement of one was suggested. Warburg and others thus considered the photosynthetic cycle as turning over at least three times as rapidly as they had proposed previously with the high rate of back reaction keeping the average energetic efficiency of the whole process just within the overall limit of 100 per cent, and concealing the one-quantum process until revealed by the device of light intermittency which was visualized as effecting sufficient time separation of the two parts of the cycle to allow for their detection.

Calvin *et al.* (9) criticized Burk & Warburg's concept of light intermittency separating partial reactions in a photosynthetic cycle by suggesting that, even taking the computed time course of gas exchange at face value, theirs is not the only possible interpretation of the manometric results. Warburg's equations (55), describing his postulated cyclic reaction mechanism in terms of gas exchange by forward and back reactions of a cycle, were found to apply as well to a very different model involving no back reactions in the Warburg sense. Presumably, the alternate model of Calvin *et al.* was not to be taken seriously for, if it is to fit the observations of Burk & Warburg, it apparently also involves a quantum requirement so low as to demand its rejection. In addition, it postulates that the oxygen production and carbon dioxide absorbing reactions are rapidly reversible. The consequences of these postulates were not explored in Calvin's brief review but are difficult to reconcile with other work cited therein.

From the standpoint of the Burk-Warburg experiments alone, it does not seem certain that the gas exchange measurements can be unambiguously interpreted in the manometric studies of Warburg & Burk and their collaborators. These reports of a one-quantum oxygen liberation or an accelerated combustion in experiments with intermittent illumination of short duration have not been confirmed outside of the laboratories of Warburg & Burk. Brackett (43) looked for such an effect with the oxygen electrode which has the advantage of much better time resolution than the manometric method and is also quite specific for oxygen. Brown (42) attempted to confirm the accelerated combustion by the use of tracer oxygen in the gas phase but not in the water of the cell suspension. Using a period of light intermittency of one minute there was no indication of enhanced rate of tracer oxygen uptake in such experiments.

The Chemical Pathway of Assimilated Carbon

The "first product" concept.—Before the advent of tracer carbon the only apparently feasible approach to the problem of identifying the so-called

"first product" of photosynthesis was to determine what substances accumulate during a period of assimilation. Such attempts were conditioned by the reasonable speculation, based on the stoichiometry of measured gas exchange, that the "first product" should be some kind of carbohydrate. Several workers had found light-dark dry weight differences in experiments on various plants to be attributable chiefly, but by no means entirely, to known carbohydrates. Using modern analytical methods, Smith (60, 61) was able to recover as carbohydrate practically all carbon assimilated by sunflower leaf tissue during light exposures of about 0.5 to over 2.5 hr. The fact that photosynthesis by mature sunflower leaf cells almost quantitatively yields carbohydrate as an ultimate translocation or storage product leads inevitably to the result obtained by Smith. Comparable studies by others on different species implicated other products as well. But there is no way to be sure that any of these substances represents a product formed immediately by the photosynthetic process, or whether the product is the end result of perhaps a long series of metabolic transformations only remotely connected with reactions of photosynthesis proper. Manifestly, the basic problem is one which cannot even be hopefully studied without employing isotopes and, since the required tracer experiments involve only brief exposures of assimilating cells to tagged CO_2, tracer dilution factors are enormous precluding the profitable use of any but radioisotopes.

The advent of radiocarbon.—Research on photosynthesis entered upon a new era with the advent of readily available, long-lived, radioactive carbon. The frequently reviewed, exciting, pioneer studies of Ruben and coworkers with C^{11} had given promise of greater things to come and, in fact, the search for C^{14} was inititated by Ruben largely for use as a tracer in photosynthetic studies. The results of Ruben's preliminary work with C^{11} were so unexpected as to excite the interest of other chemists and physiologists who renewed the attack on this problem with the much more convenient tracer, C^{14}, when it became generally available in 1946. Many students of photosynthetic problems believed, for the first time, that their goal of understanding the fundamental mechanism of photosynthesis was then in sight.

Almost overnight, interest shifted from the kinetic approach to the application of tracer methods, and a small army of biochemists, at long last provided with what seemed to be the necessary weapons, charged ahead in a frontal attack on chemical aspects of the problem. Previous kinetic studies were at first largely ignored, apparently in the belief that a delineation of the chemical pathway of assimilated carbon from CO_2 to the major organic constituents of the photosynthesizing cell would provide the clue, if not the complete solution, to the central problem of photosynthesis: how light energy is transformed into chemical energy. A healthy optimism prevailed concerning the potential fruitfulness of tracer methods combined with newly elaborated techniques such as paper chromatography.

The first results obtained with C^{14} published in the postwar era apparently were only indirectly related to photosynthesis. Studies were carried

out in Calvin's laboratory at Berkeley on the dark uptake of tracer carbon by algae which were exposed to $C^{14}O_2$ immediately after a period of illumination during which photosynthetic assimilation had been prevented by the absence of CO_2. The technique was described as "preillumination" and lately the term has also been used in publications from other laboratories with this restricted meaning.

Another research group at Chicago, comprised of Gaffron, Fager, and others, began studies of $C^{14}O_2$ incorporation during photosynthesis in experiments patterned after the earlier studies of Ruben. Subsequently, Calvin's laboratory also carried out experiments immediately concerned with photosynthesis and began a separation of products of photosynthetic assimilation of tracer carbon particularly by the green algae, *Chlorella* and *Scenedesmus*. It soon became clear that photosynthesis incorporates tracer carbon into many compounds in a very short time. Apparently, to identify a single tagged product (or intermediate) requires exposure times not of hours (Smith), or minutes (Ruben), but of seconds.

Phosphoglyceric acid as the first stable product of carbon assimilation.—From experiments in which the tagging time was 30 sec., Calvin & Benson (62) proposed that a major early product of tracer carbon assimilation was triose phosphate, since glyceric acid (presumably phosphoglyceric acid *in vivo*) was reported to be the major product in which tracer carbon was recovered. Data substantiating the identification were reported later (63). The identity of phosphoglyceric acid as the major product of very brief photosynthetic tagging experiments with algae was amply documented by Benson *et al.* (63) and confirmed by Fager *et al.* (64, 65). Additional confirmation for a higher plant was provided by Aronoff (66, 66a). The key compound probably is 2-phospho-D-glyceric acid. Although much of the tracer occurs in the 3-isomer, Calvin *et al.* (67) arrived at the provisional conclusion that the labelling of 3-phosphoglyceric acid was secondary. In view of this, it is unfortunate that the identification of the 2-isomer among the early photosynthetic products is still tentative.

Varying the time of exposure of illuminated algae to $C^{14}O_2$, the spread of tracer into a large number of compounds was studied in a series of contributions from Calvin's laboratory and by the Chicago group. These papers generally contained appropriate speculations concerning possible chemical pathways by which the tracer carbon spread into all the cellular constituents. Calvin's studies were aided by the exploitation of paper chromatography using radioautography to locate radioactive spots on the chromatograms. In addition, degradation studies on tagged compounds (e.g., phosphoglyceric acid) have provided useful information restricting speculations about probable reaction mechanisms. As new experimental results were acquired hypotheses concerning the pathway(s) of carbon assimilation have multiplied. However, conclusions which can safely be reached from incontrovertible evidence are relatively limited and it seems desirable to separate the intriguing speculations from well established facts.

At present, there is general agreement that the chief port of photosynthetic entry of tracer carbon into the metabolic machinery of the cell is the carboxyl group of phosphoglyceric acid. This suggests the existence of a 2-carbon acceptor for the $C^{14}O_2$ undergoing assimilation. The alpha and beta carbons of phosphoglyceric acid rapidly become radioactive but significantly less rapidly than the carboxyl carbon. This argues in favor of some kind of cyclic regeneration of the acceptor from assimilated carbon. Speculations concerning the postulated cycle are further restricted by the fact that the 2-carbon precursor of phosphoglyceric acid apparently must be symmetrical with respect to tracer since the alpha and beta carbons of phosphoglyceric acid acquire radioactivity at approximately equal rates. Glycine and glycolic acid are the only rapidly tagged 2-carbon compounds which have been identified on the Berkeley chromatograms. Both compounds are said to be symmetrically labelled. These compounds are thought to be related to but not identical with the 2-carbon acceptor (68, 69). Of course, the acceptor may not occur as such but only as a 2-carbon fragment of a larger molecule. There is no direct evidence concerning the identity of the acceptor and no consensus has been reached concerning its probable origin.

Appearance of tracer in compounds other than phosphoglyceric acid.—Tracer appears in pyruvate about as fast as it does in phosphoglycerate although, upon isolation, the ratio of total radioactivity in glycerate to that in pyruvate was found to exceed significantly the equilibrium value for the interconversion of these compounds, suggesting that the phosphoglycerate was not derived from pyruvate but rather the reverse (64, 70). This conclusion was further substantiated by studies with cell-free, leaf macerates described below (71, 71a). The radioactive pyruvate probably was present originally as phosphopyruvic acid. The studies reported by Fager *et al.* (64, 70) applied to tagged pyruvate obtained after hydrolysis. The Berkeley group reported radioactivity in phosphopyruvic acid and so labelled certain spots on their chromatograms, although information which supports this identification has not yet been reported in detail.

The Calvin group has also reported the very early appearance of tracer in alanine, malic acid, and aspartic acid. Other compounds become tagged much more slowly. They are thought to represent tracer incorporation via reactions not directly concerned in photosynthesis. As tagging times increase the spread of tracer into many compounds becomes accountable by a multiplicity of pathways. No particular over-all scheme seems to be especially superior to others.

It cannot be claimed that consensus in this field goes far beyond the above points. At present, there is no general agreement about the existence of ports of rapid CO_2 entry besides the carboxyl group of phosphoglyceric acid. Other primary carboxylations have been postulated without good experimental support. One such hypothesis concerns the origin of tagged malate, another early product of C^{14} incorporation. While malate (or some related C_4 product) has frequently been suggested by Calvin's group as the

product of a primary $C^{14}O_2$ fixation reaction with some C_3 acceptor, the critical experiments (72) in favor of this hypothesis were unconvincing as Gaffron *et al.* have pointed out (70). Additional evidence from Calvin's laboratory makes the hypothesis improbable with regard to malate itself (73). At the present time there seems to be no compelling reason to postulate more than one significant entry port for $C^{14}O_2$ incorporation in photosynthesis.

Dark uptake after illumination.—In early "preillumination" experiments Calvin & Benson (62, 74) reported that illuminating algae in the absence of any carbon dioxide resulted in greatly increased uptake of tracer when $C^{14}O_2$ was added in the dark period after the illumination. By varying the interval between the end of the illumination and the introduction of $C^{14}O_2$, it was possible to measure the dark uptake as a function of time and to determine the course of its decay. Curves representing the decline of the dark pickup of tracer indicate a half-life of about 4 min. although we cannot appraise the reliability of this value since the curves were given without any experimental points. These results were reminiscent of the dark pickup studied by Aufdemgarten (39) and McAlister (75, 76) but their relation to photosynthesis was initially questioned by the Chicago group (77). The "preillumination" experiments reported by Calvin *et al.* were designed with the concept that photochemical reactions of photosynthesis should be separate from the thermochemical reduction of carbon dioxide, that the light reactions produce a relatively long-lived "reducing power" which then can function in a rather nonspecific fashion. Therefore, it was not surprising that Calvin's group interpreted its results in the light of this basic assumption. For a number of reasons the Chicago group, on the other hand, considered the photochemical reduction of a carbon dioxide-acceptor complex a more likely possibility, and rejected the concept of light-generated, nonspecific reducing power. This fairly clearcut distinction between the fundamental viewpoints of the Chicago and Berkeley groups has been maintained up through the latest publications from the respective laboratories. The Chicago workers have studied dark pickup reactions systematically and have provided an interpretation of the phenomenon which differs from that of the Berkeley group.

Fager *et al.* (64) confirmed the relatively long half-life of the enhanced tracer uptake in the dark after preillumination. Two minutes was the value suggested but here, too, experimental points were not shown (78).

By measuring total $C^{14}O_2$ dark uptake as a function of time after the end of illumination, Gaffron *et al.* (70) determined the time course of tracer incorporation both after a period of steady state photosynthesis and after "preillumination" without CO_2. This dark uptake was, in both cases, cyanide sensitive and, also in both cases, the tracer was recovered almost entirely as phosphoglyceric and pyruvic acids.

The Chicago group called attention to the difference between the rapid (half-life, 3 sec.) disappearance of dark pickup activity after photosynthesis

as compared with the much slower (half-life, 2 to 4 min.) decline in such activity after "preillumination" when tested by the Berkeley method of exposing the cells to $C^{14}O_2$ for a standard interval as a function of time after the light was turned off. Their interpretation (64) was based on the difference in prevailing CO_2 concentrations in the two types of experiments. However, this explanation should be amended by noting that the different experimental procedures measured different processes. The rapid dark uptake of tracer after photosynthesis (half-life, 3 sec.) was measured as the time course of a reaction running to completion in 20 to 30 sec., viz., as interpreted by the Chicago group, the fixation of $C^{14}O_2$ by an acceptor substance produced in the light but surviving in the dark. The longer survival of dark pickup activity after "preillumination" represented, on the other hand, the time course of disappearance of surviving acceptor when no exogenous carbon dioxide was supplied.

If we assume that the Chicago and Berkeley "preillumination" experiments are in other respects comparable, the light induced ability of algae to fix CO_2 decreases more rapidly in the dark when exogenous CO_2 is present during the entire course of its decline (half-life, 15 sec.) (70, 78) than when it is supplied at various times after the end of the light period (74). It is this difference which may be accounted for by different CO_2 tensions. However, comparing by the same method the time course of dark pickup for each type of experiment, the data of Gaffron *et al.* (70) clearly distinguished the 3 sec. half-life of enhanced dark uptake after photosynthesis from a 15 sec. half-life for similarly enhanced dark uptake after "preillumination." Considering all the evidence, it appears unlikely that different CO_2 tensions (prior to adding tracer) can explain this discrepancy which is suggestive of a more fundamental difference between dark pickup of tracer after photosynthesis and tagging after "preillumination."

The Chicago group stressed the point that during the dark pickup which they observed, tracer was incorporated only into carboxyl groups of phosphoglyceric (to a lesser extent pyruvic) acid, whereas, an equivalent amount of tracer incorporation during normal photosynthesis carries much of the C^{14} beyond this initial stage. According to their view, the carbon dioxide acceptor is produced in the light and survives in the dark until it reacts with either exogenous $C^{14}O_2$ or endogenous carbon dixoide; in the former case leading primarily to carboxyl-labelled phosphoglyceric acid. The further metabolism of this compound is normally dependent upon photochemical reduction. Phosphoglyceric acid is thus considered a true intermediate of photosynthesis.

In order to arrive at the simplest reasonable explanation of these kinetic results, Gaffron *et al.* (64, 70, 78) postulated an unstable 3-carbon precursor to phosphoglyceric acid. Accordingly, the dark pickup curves represent the time course of stabilization of the precursor as phosphoglyceric acid (a dark reaction). The hypothesis is illustrated as follows:

$$[C_2] + CO_2 \rightleftarrows [C_3] \rightarrow \text{phosphoglycerate.}$$

The hypothetical precursor, $[C_3]$, was conceived as a compound which rapidly exchanges its terminal (carboxyl?) carbon with that of CO_2. Thus it could become tagged very rapidly by exchange. It is also postulated to be unstable so that, unless transformed into phosphoglyceric acid, any C^{14} it may have incorporated would be lost as $C^{14}O_2$, thus, never appearing among the isolated products of photosynthesis or after-pickup. While this hypothetical unstable 3-carbon precursor is not required as the only possible explanation of the kinetic data on dark pickup after an illumination, it does provide a simple and self consistent mechanism by which to account for the tracer incorporation under these experimental conditions.

Controversial experiments and interpretations.—The rationale of photosynthetic tagging experiments both at Berkeley and at Chicago involved the basic asssumption that tracer assimilation was carried out under steady-state conditions. That such conditions did not obtain for "preillumination" tagging experiments was, and is, a most serious impediment in the way of their proper interpretation. At both Berkeley and Chicago laboratories, the contention that tagging was carried out under steady state conditions was not at first supported by convincing evidence nor subjected to critical scrutiny. However, the basic assumption was not called into question until Calvin *et al.* published a time curve for total radioactivity assimilated by photosynthesizing algae which departed severely from linearity. The rate of tagging in the first moments was at least five times the subsequent rate (67, cf. Fig. 2). The nonlinearity was explained by suggesting that it was an initial response to increased tension of carbon dioxide; in other words, that a steady state did not prevail. The implications of such a circumstance were ignored. Utter & Wood called specific attention to these data in a critical review of carbon dioxide fixation mechanisms (79, cf. p. 137). These authors criticized the view that phosphoglycerate must be an important early product or intermediate of photosynthesis simply because it incorporates tracer initially more rapidly than other constituents. Utter & Wood elaborated on the familiar concept of accelerated tagging through exchange reactions whereby tracer could be incorporated at a rate greatly in excess of the net assimilation rate into a compound which may or may not be on the main pathway of assimilation. The time course of total assimilation under such conditions would be concave toward the abscissa and it is little wonder that Utter & Wood saw in Calvin's data vindication of their skepticism (79).

However, the experimental curves of tracer uptake with time as Calvin reported were said not to be characteristic of tagging experiments in Chicago (70), and recently Fager & Rosenberg (80) presented very convincing confirmatory data. The time course of total tracer uptake observed by them was very slightly convex toward the abscissa. Most significant was the observation that the curve for incorporation of C^{14} into the carboxyl group of phosphoglycerate was strictly linear during the first minute of tagging and extrapolated to $+1 \pm 0.5$ sec. It is thus reassuring to have the necessary

confirmation concerning the basic assumptions implied in our evaluation of the importance of phosphoglyceric acid on the main pathway of photosynthetic assimilation.

It is not certain whether the difference between the linear time course of tracer assimilation demonstrated by Fager & Rosenberg and the remarkably nonlinear curves published by the Berkeley group (67, 81) can be explained on the basis of what has been published. However, it appears that the difference may reside in whether or not truly steady-state conditions prevailed in the critical experiments. Obviously it is essential that a steady-state be maintained during tagging but this condition has not always prevailed in either the Berkeley or Chicago laboratories. Fager (64, 65) suggested that a steady-state may not have obtained in some of the early tagging experiments at Chicago. Calvin *et al.* (67) indicated the absence of steady-state conditions (changing pCO_2) during tagging in at least some Berkeley experiments. Among the earliest descriptions (May, 1948) of the Berkeley methods of photosynthetic tagging, it was reported that the algae were maintained under conditions of steady-state photosynthesis with excess carbon dioxide for a time; *the suspension was then flushed with air for 5 minutes*, and the tracer was added (82). A similar statement was made with reference to more recent tagging experiments (83, 84, 85). In other cases the details of photosynthetic tagging were not given but the experimental points show the time curve of tagging to be grossly nonlinear over its entire course in spite of the lines drawn on the graphs (72, cf. Fig. 1). In still other cases the data are fitted reasonably well by straight lines (84, cf. Fig. 1), but, in some instances where such lines can be extrapolated back to the early moments of tagging, it is clear that linear extrapolation does not run through the origin at zero time (72, cf. Figs. 1 to 4).

It is implied in various reports that tagging was carried out at Berkeley under steady-state conditions (69, 73, 86), but even in a contribution entitled *Kinetic relationships of the intermediates in steady state photosynthesis* (84) the data themselves constitute incontestable proof that serious departure from steady-state conditions occurred. In these experiments, departure from steady-state conditions due to flushing with air just before adding tracer was responsible, perhaps, for the initial surge of radioactivity into the cells. Subsequent tracer assimilation at about 40 per cent of the initial rate may or may not have been limited by carbon dioxide tension as suggested by Benson and collaborators. The *decline* in radioactivity in phosphorylated sugars, glycine, alanine after only 4 min., and in all compounds tested (except sucrose and glutamate) after 10 to 15 min. of tagging is convincing evidence of nonsteady-state conditions.

However, the shortcomings of technique apparently were later corrected by the Berkeley group in the few months prior to a subsequent report entitled *The steady state* (87). Data therein show total tracer uptake to proceed linearly over a 30 min. period of photosynthesis with no suggestion of a concavity toward the abscissa found in curves previously reported from that laboratory. This latest result is quite in line with the data of

Fager & Rosenberg (80) and satisfactorily confirms their findings. Thus, the disagreement seems to have been resolved.

The most important area of factual disagreement relating to the dark pickup experiments now lies in whether or not the tracer spreads significantly beyond the carboxyl groups of phosphoglyceric and pyruvic acids. That it should do so, seems required of a general, reducing-power hypothesis in the light of which the Berkeley results are interpreted. That it does not do so is concluded from findings published by the Chicago group. Only further studies can decide whether there is really a significant and consistent qualitative difference between the results of the two laboratories.

Results giving promise of future progress.—At this point we shall exercise a reviewer's prerogative of predicting future trends. We wish to call special attention to three quite separate developments, each of which appears to be important and promising.

(*a*) Recently, Benson *et al.* (88, 89, 90, 91) found it possible to identify as phosphates of ribulose and sedoheptulose certain previously unidentified spots on chromatograms displaying products of short term photosynthesis. Adequate localization of C^{14} within the molecules of these sugar phosphates is eagerly awaited. The existing evidence is such that the presumed photosynthetic roles of these compounds remain a matter of speculation. This subject was discussed authoritatively in a recent review (9). It is mentioned here chiefly because there is some reason to believe that these C_5 and C_7 compounds may be closely related to the much sought after 2-carbon acceptor discussed above. As yet, however, the data reported are only strongly suggestive of this possibility.

(*b*) By exposing an illuminated algal suspension to a nearly constant excess concentration of $C^{14}O_2$ for a relatively long time, Calvin & Massini (87) found that the level of incorporated tracer either reached a saturation level in a very few minutes (e.g., phosphoglyceric acid) or continued to rise with time as for a final storage product accumulating in amount during illumination (e.g., sucrose). This is a particularly noteworthy contribution for it represents the exploratory application of a technique which will allow an estimate of the prevailing sizes of metabolic pools of key compounds in photosynthetic assimilation. The abrupt changes in levels of radioactivity in phosphoglycerate and in a fraction containing mainly ribulose diphosphate upon the transition from light to dark or the reverse, are interesting since they are possibly the consequences of abrupt changes in the active pool sizes of the respective constituents. As yet this technique is only in the preliminary stage of exploitation for photosynthetic studies. However it holds forth promise of providing data on specific activities of tagged intermediates, the need for which has been stressed by Wood (92) and Utter & Wood (79). By an obvious extension of the method it is possible, at least in principle, to distinguish, for a given intermediate, between its total quantity present and the fraction which is intimately involved in photosynthesis.

(*c*) Fager (71) prepared cell-free spinach leaf macerates which exhibited

increased $C^{14}O_2$ fixation upon illumination. The rate of this light-enhanced fixation was no greater than 1 per cent of the photosynthetic rate of the original tissue under comparable conditions. The rate also decreased with time. A particularly noteworthy feature of the fixation was that the major part of the radioactivity was recovered as phosphoglyceric and phosphopyruvic acids just as in short term photosynthesis with intact cells. Other reports (93, 94, 95, 96, 97, 98) of photochemical CO_2 fixation *in vitro* (accomplished by coupling known reductive carboxylations to a Hill reaction) do not provide for the incorporation of C^{14} in a pattern like that of photosynthesis.

Fager (71a) achieved some fractionation of his preparation by acetone precipitation. The proteinaceous precipitate from 47.5 to 60 vol. per cent acetone contained over one-half of the original activity and about two-thirds of the activity responsible for $C^{14}O_2$ fixation as phosphoglyceric and phosphopyruvic acids. The concentration of activity achieved over that of the original spinach juice preparation was not specified but apparently it represented a considerable purification in terms of either dry weight or total protein. Tracer fixation in the chlorophyll-free "purified" fraction was not photosensitive.

In both crude and "purified" preparations Fager concluded that tracer fixation in phosphoglycerate apparently preceded that in phosphopyruvate. Ratios of total activity in phosphoglycerate to that in phosphopyruvate were as high as 10:1. In the case of the "purified" fraction, ratios of 12:1 and 19:1 were reported. Fluoride ($10^{-2}M$) which should inhibit effectively the enolase promoting equilibration of these compounds, was found to increase the ratio to 72:1. These results confirm similar findings with regard to activity ratios of these compounds tagged by short term photosynthesis.

Fager tested various additives on the purified fraction. Adenosinetriphosphate ($10^{-3}M$) only reduced total fixation. Arsenate ($2\times10^{-3}M$) caused a small shift in fixation from ether-extractable acids to neutral substances. Ribose-5-phosphate ($1.5\times10^{-3}M$) and phosphoglyceric acid ($10^{-2}M$) were strongly inhibitory. Sedoheptulose ($1.5\times10^{-3}M$) was without significant effect. Glyoxal ($10^{-4}M$) also was inhibitory contrary to its stimulative effect on unfractionated material. Glyoxal and other 2-carbon compounds were reported (71) to enhance fixation by crude preparations but, in view of the anomalous results with glyoxal, the influence of other 2-carbon compounds on the activity of purified preparations cannot easily be predicted. Cysteine ($2\times10^{-2}M$), when present during the fractionation, enhanced the activity several fold.

The activity of Fager's preparations can be explained if it is assumed that they contain traces of the normal 2-carbon acceptor functional in photosynthesis and that more acceptor is produced at a slow rate in the light. The very low activity of the preparations relative to comparable photosynthetic fixation may be due either to rate limitation imposed upon reactions involving production of the acceptor or to reactions relating to the

oxidation of the primary photochemical oxidant to molecular oxygen. Since these spinach juice preparations are not active in a conventional Hill reaction, the latter alternative is deemed likely.

While the CO_2 fixing ability of Fager's preparations was very low it seems possible that modifications in the preparatory procedure may yield cell-free material more active in both $C^{14}O_2$ fixing ability and photochemical oxygen liberation, a hope which encourages continued study of this approach to photosynthesis *in vitro*.

In appraising the current status of our knowledge of the chemical pathway of carbon assimilation, it is clear that work with tracer carbon has produced, during its short and turbulent history, a wealth of information which is just beginning to assume some aspects of coordination. These results are both quantitatively and qualitatively encouraging. On the other hand, real progress toward an understanding of the fundamental mechanism of photosynthesis has been somewhat less than most of us would have predicted. New techniques bring new problems and in photosynthesis, as in other research fields, it has been demonstrated that one can arrive at wrong conclusions even more easily using tracers than without their aid.

Enzymes in Photosynthesis

The following section will deal primarily with the existing evidence for the participation of certain enzymes and enzyme systems which have been postulated to play a role in photosynthesis. Most of the information has been derived from studies on the intact photosynthetic apparatus or from studies on partial reactions of photosynthesis, as in the Hill reaction and its various modifications. Partial systems, such as the Hill reaction, most likely should be considered as multienzyme systems in the sense of Dixon (99). Actually, little information is available on specific enzyme systems in photosynthesis, but an effort is made to survey our present knowledge of such systems. Enzymes which are concerned with the glycolytic transformation of phosphoglyceric acid into respiratory intermediates and sugars will not be dealt with in this review, even though it is well realized that some of the intermediates of glycolysis may be of importance as precursors in the reaction system involved in photosynthetic carbon dioxide fixation.

Catalysts of the photochemical system: chlorophyll as hydrogen donor.—Chlorophyll may be considered as a photocatalyst concerned with the transformation of light energy into chemical energy in the process of photosynthesis (44). The role of chlorophyll and of accessory pigments in the process of energy transfer has been reviewed by Franck (7, 100), and by Rabinowitch (1, 3) who also has reviewed the theories of photochemical oxidation and reduction of chlorophyll during photosynthesis (44, pp. 554–57). At this point, only the evidence for and against hydrogen transfer by chlorophyll will be reviewed. Franck & Herzfeld (101) had postulated that chlorophyll in photosynthesis oscillated between an oxidized and reduced form, denoting the reduced form as H-chlorophyll. Norris *et al.* (102) tested the

hypothesis that a proton was involved in such a postulated reversible oxidation reduction cycle by incubating *Chlorella* cells in tritium water in the dark and in the light. No incorporation of tritium into chlorophyll could be detected. These investigators, however, pointed out that isotope discrimination between hydrogen and tritium might be so large that the experiment should be carried out at high concentrations of deuterium water. Norris *et al.* also demonstrated the absence of thermal exchange between purified chlorophyll and tritium water. The experiments *in vivo* were repeated by Calvin & Aronoff with heavy water, and the incorporation of C^{14} into the chlorophyll of algae was studied simultaneously (103, cf. 104, p. 559). They reported that only one fourth of the theoretically expected amount of deuterium was incorporated into chlorophyll, which they believed could be attributed to synthesis of chlorophyll. Frenkel (105) has tested the procedure of Calvin & Aronoff to determine possible exchange of hydrogen isotopes during the extraction of chlorophyll. *Scenedesmus* cells incubated in the dark were extracted with acetone containing heavy water. Also under these conditions no incorporation of deuterium into chlorophyll was observed. It has been suggested that there is a large reservoir of nonexchangeable hydrogen which is acted upon photochemically (44, 104). The data on hand do not permit a conclusion as to the feasibility of this hypothesis.

Another suggestion is that the extracted chlorophyll is identical with the oxidized rather than with the reduced form of chlorophyll (44). This may well be possible, judging from experiments on reversible bleaching of chlorophyll (106). It is not known, however, whether this reaction involves transfer of protons.

Calvin & Aronoff (103) have suggested that chlorophyll functions via an enolizable hydrogen. The experiments of Weigl & Livingston (107), however, appear to speak against such an assumption. It has also been proposed that only a small part of all the chlorophyll molecules are active in photosynthesis. This is unlikely because of the high efficiency of photosynthesis at low light intensities. Weigl & Livingston have redetermined the possibility of an exchange reaction between chlorophyll and heavy water in a number of solvents and could not observe any exchange.

Weigl & Livingston (108) also studied the possibility of hydrogen transfer in the chlorophyll sensitized reduction of butter yellow by deuterated ascorbic acid in dry dioxane. When four to seven butter yellow molecules had reacted for each molecule of sensitizer present, with about 75 per cent reduction of the butter yellow, the chlorophyll was separated and analyzed for deuterium. On the assumption that chlorophyll contained one active hydrogen which could be transferred, and that one hydrogen would be transferred for each molecule of butter yellow reduced, not more than 4 per cent of the expected amount of deuterium could be detected in chlorophyll. These authors conclude that in this particular reaction hydrogen transfer is not involved, but point out that this may not be necessarily true for other chlorophyll sensitized reactions.

In conclusion it may be said that we have no evidence that chlorophyll transfers protons in photosynthesis. The experiments performed thus far have not tested all the postulated reaction mechanisms, and a possible proton transfer by chlorophyll may have eluded investigators to the present time.

Catalysts of the photochemical system: catalyst B of Franck & Herzfeld.—In an effort to establish a consistent theory of photosynthesis which would take into account all of the available kinetic data, Franck & Herzfeld (101, 109) postulated a catalyst active in the stabilization of intermediate labile photo products (catalyst B). This catalyst accounts for the magnitude of flash saturation when only about one chlorophyll molecule out of a thousand is absorbing light, and under some conditions for the value of the saturation rate of photosynthesis in continuous light. From a consideration of the high quantum yield of photosynthesis at low light intensities and because of the absence of long induction periods, Franck & Herzfeld concluded that photosensitive substrate was probably present in a concentration equal to that of chlorophyll. Franck & Herzfeld then postulated that unstable photoproducts are formed by the photochemical reaction and the capacity of catalyst B to stabilize these products determines the amount of photosynthesis per flash at saturation. At the same time back reactions are postulated to occur which transform the unstable photoproducts to their original energy level in case catalytic stabilization does not keep pace with their formation. At low light intensities, catalyst B is effective in stabilizing practically all unstable photoproducts; back reactions become important only at higher light intensities (near and beyond light saturation). Another consequence of this theory is the interpretation of the effect of cyanide on the yield per flash in Emerson & Arnold's experiments (110). The yield per flash is independent of cyanide concentration if the period between flashes is made long enough. Weller & Franck (111) have repeated these experiments in more detail. They interpret their results to mean that cyanide has no effect on the working period of catalyst B; however, cyanide affects the rate of formation of photosensitive substrate (inhibition of catalyst A of Franck & Herzfeld) Rieke & Gaffron (112) also have demonstrated in more intricate flashing light experiments that the catalyst which limits photosynthesis per flash at light saturation is not the cyanide sensitive catalyst of photosynthesis. There are no known inhibitors of catalyst B.

Clendenning & Ehrmantraut (113) studying the photochemical reduction of Hill reagents by isolated chloroplasts measured the working period of the dark catalyst as about 1/100 sec., thus supporting Franck & Herzfeld's view that the catalyst contributing to flash saturation is not concerned with reactions immediately involved in carbon dioxide fixation.

Enzymes and co enzymes involved in transfer of reducing energy to Hill oxidants.—The discovery that isolated chloroplasts in the presence of pyridine-nucleotides and the proper enzymes and substrates will carry out reductive carboxylation (93, 94, 95, 96, 97, 98) has lent strong support to the view of either coenzyme I or II, or both, being the natural Hill oxidants

in photosynthesis (114, 115, p. 586). This concept, however, has been criticized by Franck (7, 100) and by Tolmach (96). Franck has pointed out that the natural oxidant in photosynthesis has to be present in high enough concentration to suppress the photochemical reduction of molecular oxygen, which was demonstrated by Mehler (116) to be an effective Hill oxidant. The concentration of pyridine-nucleotides in leaves, as determined thus far, seems to be several magnitudes smaller (117). Furthermore, the reduction of pyridine-nucleotides in chloroplast preparations occurs only with small yields, and no direct spectroscopic reduction has been observed (116). Also, there is no evidence of the normal Hill reaction requiring either coenzyme I or II.

Ehrmantraut & Rabinowitch (16) have observed an inhibition by malonate of the photochemical reduction of quinone by intact *Chlorella* cells. This inhibition is reversed by an equimolar amount of fumarate, thus pointing to an inhibition of succinic dehydrogenase. The authors point out that this conclusion is contrary to the hypothesis of Franck (7, 100) which assumes a direct reduction by chlorophyll of such oxidants as quinone. Ehrmantraut & Rabinowitch cite the possibility of an unspecific narcotization by malonate, but no further experimental evidence is available on this point. Other enzyme systems are inhibited by malonate besides succinic dehydrogenase (118), although the latter enzyme seems to be the one most sensitive to this inhibitor. The effect of malonate on intermediates in photosynthesis was studied by Bassham *et al.* (73) who could not find any effect on the formation of phosphoglyceric acid, but observed an inhibition of the formation of C^{14}-labelled malic acid.

The effect of a number of other metabolic inhibitors on the Hill reaction and its modifications has been studied (16, 119, 120). Spikes *et al.* (121) have studied the effect of univalent ions on the photochemical activity of isolated chloroplasts. A number of univalent anions act as noncompetitive inhibitors; fluoride, however, gave a different type of inhibition than any other of the univalent ions tested (122). No critical analysis is available to indicate at which points these inhibitors interfere in this complex reaction system.

Enzymes involved in carbon dioxide fixation.—The initial fixation of carbon dioxide has usually been assumed to be a carboxylation reaction. Kinetic data obtained prior to the use of tracer carbon as well as results of isotope experiments are consistent with the hypothesis that this carboxylation reaction is inhibited by cyanide. In the theory of Franck & Herzfeld (101, 109), this cyanide-sensitive enzyme system has been referred to as "catalyst A."

Specific enzyme systems suggested to take part in carbon dioxide fixation are coenzyme-linked systems active in reductive carboxylation (114), and carbonic anhydrase which has been implicated at various times to take part in the photosynthetic sequence of reactions (44, 123).

Enzymes involved in carbon dioxide fixation: carboxylases.—The topic of "carboxylating enzymes in plants" has been reviewed recently (124). Only certain problems relating to photosynthesis will be discussed here. Of the

known carboxylases, the bacterial amino acid decarboxylases, particularly glutamic acid decarboyxlase, shows high sensitivity toward cyanide (125). For other known carboxylases no high sensitivity to cyanide has been established except for the "malic" enzyme system discussed below.

Clendenning *et al.* (126) have looked for an enzyme in plant extracts decarboxylating phosphoglyceric acid and have found no evidence for direct decarboxylation or indirect decarboxylation through pyruvate. They also have studied the relative distribution of different carboxylases in plants and demonstrated a much higher abundance of the malic system in green tissues in contrast to nonphotosynthetic tissues. In their test system involving reduced TPN[2], pyruvate, carbon dioxide, and "malic" enzyme they could not demonstrate any cyanide sensitivity as long as the enzyme was present in excess. At low enzyme concentrations $5\times10^{-4}M$ HCN[2] produced a definite inhibition. This observation is of interest even though we have no direct evidence for the participation of the above system in photosynthesis.

In the cell free system prepared by Fager (71, 71a), described above, the addition of DPN[2] or TPN had no effect on the light fixation of carbon dioxide into phosphoglyceric acid.

Enzymes involved in carbon dioxide fixation: carbonic anhydrase.—Most of the work on this enzyme relating to plant material has been reviewed by Weier & Stocking (127, p. 67) and by Rabinowitch (44). Since then several papers on the isolation and properties of carbonic anhydrase from plants have been published (128, 129, 130, 131, 132). A recent comprehensive review of the properties of the enzyme was prepared by Roughton & Clark (133).

Experimental evidence strongly favors the view of carbon dioxide penetrating most readily into living plant cells in the anhydrous form (44), although Steemann Nielsen (134, 135) has presented evidence for uptake of bicarbonate ion by several water plants. Österlind (136, 137) has reported utilization of bicarbonate ion by *Scenedesmus quadricauda* to be more effective under certain conditions than the utilization of anhydrous carbon dioxide. Some of these results have been discussed by Steemann Nielsen & Kristiansen (138).

Waygood & Clendenning (123, 139) critically examined conflicting evidence in the literature on the distribution of carbonic anhydrase within plant cells. They concluded from their experiments that the enzyme is localized in the cytoplasm. Furthermore, the enzyme is generally present in photosynthetic tissues and is usually absent in nonphotosynthetic tissues of the same plants. The measured activity of carbonic anhydrase (139) would be sufficient to hydrate carbon dioxide in the cell at rates comparable to those of maximum photosynthesis, based on the calculations of Burr (140). It is difficult to understand, however, why such hydration should be required in photosynthesis, in view of the evidence of anhydrous carbon dioxide being produced by a number of carboxylases (128, 141, 142).

A good deal of the evidence for participation of carbonic anhydrase in photosynthesis is based on the cyanide sensitivity of carbonic anhydrase

and of photosynthesis in certain organisms. Wood & Sibly (130) from a survey of the literature have noted a good deal of variation in the cyanide sensitivity of plant carbonic anhydrase preparations which have been examined. Differences in the cyanide sensitivity of photosynthesis in a variety of organisms could then be possibly explained by differences in the sensitivity of their carbonic anhydrases.

Wood & Sibly (130) have pointed out a similar sensitivity to azide for most plant preparations of carbonic anhydrase tested; this observation, however, may be fortuitous as the pH was not given by all investigators. Gaffron (143, p. 491) has reported an azide inhibition of photosynthesis, but it is not indicated which partial reaction is affected. Arnon & Whatley (144) and Macdowall (120) have reported azide inhibitions of photochemical reactions of chloroplasts. Thus, the available studies on azide inhibition of photosynthesis appear to contribute little to the question of participation of carbonic anhydrase in this process.

Sulfanilamide which inhibits carbonic anhydrase (133), but apparently is not specific for this enzyme as it inhibits "Zwischenferment" (145) and possibly other enzymes, does inhibit carbonic anhydrase from *Tradescantia* (139). Sulfanilamide also inhibits photosynthesis of *Chlamydomonas*, affecting a dark reaction as judged from the shape of the light saturation curve (105).

The evidence for participation of carbonic anhydrase in the photosynthetic process is thus based on its general abundance in green tissues and its sensitivity to certain metabolic inhibitors which unfortunately are not specific for this enzyme.

Enzymes relating to precursors of molecular oxygen: catalyst C.—Franck & Herzfeld (101) proposed this name for the catalytic system which is active in the liberation of molecular oxygen from an intermediate peroxide. Gaffron (146) suggested that more than one enzyme must be involved in the reaction path from primary photochemical oxidant to molecular oxygen. This assumption was based on the differential action of inhibitors on photosynthesis and photoreduction of anaerobically adapted algae. One proposed enzyme is involved in the transformation of the primary photochemical oxidant into an intermediate oxidant, which is not a photo-peroxide, and the other enzyme or enzymes catalyze the transformation of this intermediate into a hypothetical peroxide and finally into molecular oxygen. Gaffron (147) hypothesized that the oxygen liberating system is activated by light. This hypothesis has been used by Weller & Franck (111) to explain the nature of hydroxylamine inhibition of photosynthesis. The free enzyme, not inhibited by hydroxylamine, will be activated by light in the same proportion at each light intensity as the enzyme in a system not subjected to this inhibitor. Weller & Franck believe more experimental evidence should be adduced to confirm this hypothesis.

Enzymes relating to precursors of molecular oxygen: catalase.—Catalase has often been implicated as the "oxygen liberating enzyme" of photosyn-

thesis. Rabinowitch (44) has reviewed the earlier literature and feels the evidence is not in favor of such an hypothesis, but adds that the problem should be reinvestigated.

The strongest argument against the catalase theory is found in experiments by Gaffron (148), in which catalase activity in a certain strain of *Scenedesmus* was completely inhibited, while photosynthesis was not appreciably affected. Cyanide concentrations which strongly inhibit catalase activity also have little effect on the photochemical evolution of oxygen (16, 149). Tamiya (150) has criticized the objections raised against the catalase theory. He has pointed out that the measurement of hydrogen peroxide decomposition by photosynthetic tissues may not be a true measure of the catalase present in the photosynthetic apparatus. He cites experiments in which an increased catalase activity can be observed after heating of living cells; consequently, catalase activity and photosynthetic activity of living cells should not necessarily go hand in hand. Mehler (116) has raised the same point and set out to determine if hydrogen peroxide is actually produced during photosynthesis. He employed the reaction system of Keilin & Hartree (151) in which ethyl alcohol is peroxydatively oxidized to acetaldehyde by hydrogen peroxide in the presence of excess catalase. Chloroplasts, actively producing oxygen in the light in the presence of Hill reagents, will not produce acetaldehyde when excess ethyl alcohol and catalase are added. In the absence of Hill reagents, however, and in the presence of oxygen acetaldehyde is produced. This observation led Mehler to the discovery of molecular oxygen as an effective Hill oxidant under the appropriate conditions. Hydrogen peroxide does not appear to be an intermediate of photosynthesis or of the Hill reaction under conditions where other oxidant is present. Catalase from plant sources has been isolated in highly purified form by Galston *et al.* (152).

Enzymes relating to precursors of molecular oxygen: hydrogenase.—Hydrogenase by definition is an enzyme which reversibly catalyzes reactions involving molecular hydrogen. Whether hydrogenase can catalyze direct reduction of metabolic substrate by molecular hydrogen or always requires intermediate enzymes remains to be elucidated. Conversely, hydrogenase is concerned with the evolution of molecular hydrogen from certain substrates, probably not directly but as the terminal enzyme of a system active in hydrogen transport. It remains to be seen whether there is more than one enzyme which fits the above definition.

The enzyme has received a good deal of attention in the last several years and our knowledge of its properties has been reviewed by Umbreit (153) and by Gest (154). Recent work on requirements of growth factors which appear to be necessary for the production of hydrogenase in microorganisms has been reported by Billen & Lichstein (155).

Hydrogenase is present among the photosynthetic organisms in the purple bacteria (44), very probably in the green bacteria (156), and in *Rhodomicrobium vaniellii* [an unassigned photosynthetic organism (156a)],

and in certain algae (146, 157). Its presence has been reported in higher plants (158), but this observation has not yet been corroborated by other workers. There is no evidence of an essential role of hydrogenase in the "normal" process of photosynthesis in which molecular oxygen is liberated, or in bacterial photosynthesis when hydrogen donors other than molecular hydrogen are consumed in the photosynthetic process.

In algae, hydrogenase, strictly speaking, is an "adaptive enzyme." Gaffron demonstrated, in the green alga *Scenedesmus*, that incubation in hydrogen in the dark from one to several hours is required and even then complete activation may not be achieved unless the algae are carefully manipulated. No investigations are available so far to indicate the nature of the precursor of the active enzyme.

Hydrogenase from *Rhodospirillum rubrum* has been partially purified by Gest (159).

Other enzymes: cytochrome-f.—Hill & Scarisbrick (160) discovered a new cytochrome component present only in photosynthetic tissues which they named cytochrome-*f*. Davenport & Hill (161) have described the properties of this component in more detail. Its electrode potential between pH 6 and 7.7 is 0.365 v. which is slightly more oxidizing than the potential of cytochrome-*c*. Davenport & Hill have pointed out that cytochrome-*f*, in many of its properties examined thus far, is very similar to cytochrome-*c*. They have demonstrated a similarity of the bonding of the heme to protein, a considerable likeness in their spectra; also both cytochrome components in the ferro form are unable to combine at pH 7 with oxygen or carbon monoxide. Cytochrome-*f*, however, is more readily heat denatured than cytochrome-*c*.

Hill (8, 162) has made the suggestion that cytochrome-*f* may play a role in photosynthesis, because of its general presence in photosynthetic tissues, and its localization within the chloroplasts. Davenport & Hill (161) have indicated that its chemical energy could possibly be utilized in photosynthesis in a stepwise manner. On the basis of Strehler's evidence (163) that ester phosphate is formed and utilized at an accelerated rate during photosynthesis, one might postulate that cytochrome-*f* could play a role in oxidative phosphorylation. The potential between cytochrome-*f* and the oxygen electrode corresponds to a free energy change per electron which is approximately equal to the free energy of hydrolysis of an energy rich phosphate. Thus, one mole of ester phosphate could possibly be formed per mole of cytochrome oxidized. One might speculate, on the other hand, that cytochrome-*f* could play a role in the oxygen liberating system.

Thus far, at any rate, no direct experimental evidence appears to be available which points to the participation of cytochrome-*f* in photosynthesis.

Photosynthesis and Phosphorus Metabolism

Most of the work summarized here covers the last two years. The reader is referred to a number of reviews dealing with the literature previous to

1950 (12, 44, 154), also to a review on the metabolism of phosphorylated compounds in plants (164), and a recent monograph on phosphorus metabolism (165).

The most important contributions to this field have been those of Benson & Calvin and of Fager & Gaffron and their collaborators on phosphorylated intermediates in photosynthesis, and the work of Kandler (166), Strehler (163, 167) and of Goodman *et al.* (168) on transformation of phosphorus fractions during photosynthesis. The work of Benson, Calvin *et al.* and of Fager *et al.*, discussed in the preceding section, which led to the discovery of phosphorylated intermediates in photosynthesis, has laid the groundwork for further studies relating phosphorus metabolism and photosynthesis. Thus far, however, nothing is known about the reactions by which phosphorus enters into phosphoglycerate, ribulose-phosphate, or seduheptulose-phosphate which have been suggested as playing an active role in photosynthesis.

The transformation of orthophosphate during photosynthesis has been studied by Kandler (166), who worked with *Chlorella pyrenoidosa*, suspended in a phosphate free medium. Kandler observed definite changes in cellular orthophosphate levels upon illumination and when the cells were returned to darkness. The most striking changes were observed during the first minute of illumination when induction phenomena, ordinarily observed in measurement of gas exchange, also were reflected in changes of orthophosphate concentrations. The level of orthophosphate dropped sharply immediately upon illumination and rapidly recovered to a new steady-state; this steady-state value in the light was never higher than the one in the dark. When illumination was stopped the phosphate level rose to a maximum after 1 1/2 min. and rapidly fell again to a new steady-state. Kandler saw proof in these experiments that part of the light energy in photosynthesis is transformed into energy-rich phosphate. Kandler proposed two hypothetical schemes to account for the role of phosphorylation in photosynthesis. The first one postulates the degradation of the primary photochemical reductant to a reductant of intermediate energy level with the production of one energy-rich phosphate per molecule of reductant. The intermediate reductant and the energy-rich phosphate are then utilized in the process of carbon dioxide reduction. The second scheme of Kandler proposes a degradation of a fraction of the primary reductant to energy-rich phosphate, and this energy-rich phosphate in combination with the primary reductant is active in carbon dioxide reduction.

Strehler & Totter (167) have developed an elegant method for the determination of adenosinetriphosphate (ATP) by means of measurement of the luminescence of firefly extracts, and this method was applied to a study of ATP[a] levels in *Chlorella* cells under a variety of conditions (163). Strehler observed that upon illumination of anaerobically incubated cells the ATP level rose to a maximum within one minute and then fell off to a steady-state. When oxygen was admitted in the dark to anaerobically incubated cells, the ATP level rose to a steady-state value in about 30 seconds; this value at

25°C. was always higher than the ATP level in the light. However, at 4°C. the steady state value of ATP in the light was higher than in the dark in the presence of oxygen. Strehler has interpreted this observation to the effect that at low temperatures the aerobic production of ATP by *Chlorella* is inhibited to a greater extent than the light-induced formation of ATP; the rate of ATP formation (of cells previously incubated under anaerobic conditions), measured after 25 sec. illumination, saturated at high light intensities. The steady-state level of ATP, however, measured after 10 min. of illumination, reached a maximum and fell off at higher light intensities. The last two results were obtained whether carbon dioxide was present or not. When, after several minutes of illumination, the cells were returned to darkness a rapid rise in the ATP level could be observed. In the presence of carbon dioxide it levelled off within 3 min.; in the absence of carbon dioxide, however, it continued to rise for some time. Strehler has calculated a minimum ratio of 1:6 for energy-rich phosphate (as terminal phosphate of ATP) formed per molecule of oxygen produced during photosynthesis, but suggested that the actual value for the rate of formation of energy-rich phosphate may be considerably higher. Strehler proposed that ATP is generated and utilized by "light driven" reactions. Because the presence or absence of carbon dioixde had little effect on the steady state level of ATP in the light, Strehler believed that ATP is not directly concerned with CO_2 fixation but rather with the production of a reductant of the oxidation reduction level of carbohydrate. His theory otherwise appears to be similar to Kandler's first scheme of phosphate metabolism in photosynthesis.

It is of interest that in Kandler's and in Strehler's experiments the known induction phenomena in photosynthesis were reflected respectively in the inorganic phosphate levels and in the levels of ATP. In fact, Kandler's curves for phosphate levels are more or less the mirror image of Strehler's curves for ATP levels.

Goodman *et al.* (168) have studied incorporation of radiophosphate in the light and in the dark into a number of phosphorylated compounds of *Scenedesmus obliquus.* They found a rapid incorporation of P^{32} labelled phosphate into ATP in the dark and a rapid incorporation of P^{32} into phosphoglyceric acid in the light. From the rate of incorporation of P^{32} into ATP they concluded that under their conditions ATP was the first detectable product formed from inorganic phosphate.

Experiments by Kamen & Spiegelman (169) and by Gest & Kamen (170) with labelled phosphate had demonstrated that in *Chlorella* and in *Rhodospirillum rubrum* the phosphorus turnover in the fraction insoluble in trichloracetic acid (TCA) is greater in the light than in the dark, and they also had observed that light stimulated the flow of low specific activity phosphate from the insoluble phosphate into soluble phosphate, with a simultaneous flow in the opposite direction. Kamen & Spiegelman considered their observations as strong evidence that "phosphorylation is mediated by light directly or indirectly in photosynthetic organisms." Simonis & Grube (171)

could not confirm the increased uptake of labelled phosphate into the TCA-insoluble fraction by leaves of *Helodea densa.* They observed an increased uptake of P^{32} into the TCA-soluble fraction in the light which was greater in the presence of carbon dioxide than in its absence. They also observed that the level of inorganic phosphate drops in the light. Wassink *et al.* (172) on the other hand have obtained results similar to those of Kamen & Spiegelman. They found in *Chlorella* an increased conversion of TCA-soluble phosphate into TCA-insoluble phosphate in the light, particularly in the absence of carbon dioxide. In the absence of carbon dioxide they observed a conversion amounting to 30 per cent of the phosphate content of the cells into the TCA-insoluble fraction. Wassink *et al.* (173) noticed an increased uptake of orthophosphate by cells in the light in the absence of carbon dioxide. They fractionated (5 min. hydrolysis with *N* HCl at 100°C.) the TCA-soluble phosphate into a labile fraction and a stable fraction and found that the conversion of the labile fraction into the stable fraction was reduced in the presence of carbon dioxide. Addition of glucose had quantitatively the same effect as carbon dioxide in diminishing the conversion of labile into stable phosphate. Similar observations were made by Wintermans & Tjia (174), who concluded that the extra phosphate which was taken up in the absence of carbon dioxide was largely stored in labile phosphate compounds.

Holzer (175) has found that a great part of the TCA-soluble, 7 min. hydrolyzable, phosphate in *Chlorella* is metaphosphate and has suggested that the ester phosphate fraction may be small. He observed differences in the amount of metaphosphate in the light and in the dark. However, it is not apparent from the data if these differences were significant.

Phosphorylation in cell-free preparations in the light has been demonstrated by Vishniac & Ochoa (176) who incubated spinach chloroplast preparations with DPN, ATP[2], labelled phosphate, and mitochondria. Radioactive phosphate was incorporated into ATP, the incorporation depending upon the complete system and light and was active with mitochondria from either plants (mung bean) or animals (rat).

In the cell-free system of Fager (71a) described previously, addition of ATP actually caused an inhibition of carbon dioxide fixation into phosphoglyceric acid in the light and in the dark. This inhibition, Fager suggested, possibly may have been caused by ATP promoting other carbon dioxide fixations reactions, or it may have been due to ATP having reduced the amount of precursor which could be carboxylated to form phosphoglyceric acid. Fager has indicated that acylphosphate bonds were not involved in the fixation of carbon dioxide into phosphoglyceric acid in this sytem.

In conclusion, it may be said that the investigations of Strehler clearly show that oxidative production and utilization of energy-rich phosphate (as ATP[2]) are accelerated during photosynthesis. From the studies reviewed it does not seem possible to say as to whether production of energy-rich phosphate is tied to reactions involving the primary reductant of photosynthesis. Neither do we know as yet of the possible fate of energy-rich phosphate in

photosynthetic carbon dioxide fixation. Nonphotochemical metabolic transformations appear to be accelerated during photosynthesis. Thus, the increased rate of production and consumption of energy-rich phosphate may be a reflection of this increased metabolism, which, strictly speaking, may not be part of photosynthesis. The work of Fager on cell-free preparations which are capable of fixing carbon dioxide into phosphoglyceric acid would indicate that ATP is not necessary for such fixation. However, no final conclusion can be drawn from these experiments, as the activity of this system is small compared to normal photosynthesis.

The effect of certain metabolic inhibitors on photosynthesis: inhibitors which in other systems, are known to interfere with the metabolism of energy-rich phosphate.—Loomis & Lipmann (177) observed that low concentrations of 2,4-dinitrophenol (DNP)[2] would inhibit the formation of energy-rich phosphate bonds in rabbit kidney homogenates without affecting oxidation and would stimulate oxidation, in phosphate-deficient systems. In the meanwhile, this observation has been extended to numerous organisms and tissue preparations. Substances other than DNP will produce similar effects, although in each case the mechanism of this action is far from elucidated (115, p. 578).

No systematic study has been carried out on the effect of substances which might interfere with the metabolism of energy-rich phosphate in photosynthesis. Gaffron (178) demonstrated that photosynthesis and photoreduction in *Scenedesmus* (strain D-3) were strongly inhibited by DNP. Dark production of molecular hydrogen was completely inhibited by $2\times10^{-4}M$ DNP (pH 6.2) although light production of hydrogen was observed under these conditions (178a).

Holzer (175) has studied the effect of DNP on photosynthesis and respiration of *Chlorella pyrenoidosa* and obtained results similar to those of Gaffron. Holzer concluded that ATP is necessary for photosynthesis but did not report any analyses indicating an effect of DNP on the distribution of phosphate fractions. According to Macdowall (120), the photochemical reduction of indophenol by isolated chloroplasts is inhibited by low concentrations of DNP.

Azide, which has been shown to interfere with transfer of energy-rich phosphate in yeast fermentation (179), also has been shown to be inhibitory to photosynthesis (143, 180), and various degrees of inhibition of the Hill reaction have been observed (16, 120, 144). No evidence has been published, however, which would indicate that either DNP[2] or azide are inhibitory because of their interference with phosphate metabolism in these systems.

Gest & Kamen (170) made the interesting observation that in *Chlorella* the turnover of P^{32} was inhibited in the presence of cyanide in the light and in the dark. Photosynthesis was strongly inhibited; respiration, however, was stimulated by the cyanide concentrations employed. The dark effect is thus suggestive of the action of DNP described by Loomis & Lipmann (177).

Warburg (56) has reported a reversible arsenate inhibition of photosynthesis. He interpreted this effect as an inhibition of normal ATP formation. Fager (71a) has studied the effect of arsenate in the cell-free preparation mentioned in the preceding section, and actually observed a slight stimulation of carbon dioxide fixation into phosphoglyceric acid.

It is apparent from the evidence cited, that work with inhibitors which are known to interfere with the metabolism of energy-rich phosphate in certain systems, thus far has thrown little light on the problem of formation and utilization of energy-rich phosphate in photosynthesis.

The behavior of plants grown on phosphorus-deficient media.—Pirson (181) has described an inhibition of photosynthesis in *Ankistrodesmus* cultures grown on phosphorus-deficient media. This inhibition was only apparent at saturating light intensities, and it was readily reversed by the addition of phosphate within one hour. Lindeman (182) has obtained similar results with *Lemna minor* and he observed that addition of phosphate to phosphorus-deficient cultures brought about an increase in photosynthetic rates which were not accompanied by an increased chlorophyll concentration. These authors have stated that the phosphate effect described must be on a dark reaction of photosynthesis.

The Source of Photosynthetic Oxygen

Fundamental to our understanding of the mechanism of photosynthesis is the source of the evolved oxygen. Does it come from water, carbon dioxide, or both? This matter has been brought up in several reviews (183, 184) and treated in a fashion which seems to us not altogether satisfactory. Reviewers and authors of original research contributions (86, 185) habitually cite the initial application of the tracer technique to this problem by Ruben *et al.* (186) and imply that the matter was then settled. This work has even been presented for consumption by beginning students in elementary text books (187, 188, 189). Without deprecating the ingenuity of Ruben and his co-workers in their pioneer use of isotopically enriched oxygen or disputing the validity of their very clear cut experimental results, it should nevertheless be pointed out that their experiments were in no sense conclusive and their results were admittedly quite ambiguous. In fact, one of the authors of the original work has retracted any claim to the contrary and it may be worthwhile to quote from the discussion of Kamen & Barker (190) their comments on the earlier conclusion that water rather than carbon dioxide was the ultimate precursor of photosynthetic oxygen:

> Such a conclusion is reasonable, but by no means certain since it depends on the unproven assumption that isotope exchange is no more rapid inside the cells . . . than in the outside medium . . . ." "It is quite possible and, indeed, even probable that this assumption is incorrect. . . . " "It can be calculated that at pH 6, the randomization of O^{18} is rapid enough to invalidate the conclusion that carbon dioxide is not a source of oxygen." " . . . These experiments do not provide proof of the role of water as the [sole] precursor of oxygen.

The difficulty was believed to be circumvented by employing the oxygen isotopes at their equilibrium concentrations for the reaction, $H_2O^* + CO_2 \leftrightarrows H_2O + CO_2^*$, which leads to a slight but significant enrichment of the heavy oxygen isotope in the CO_2. This was done by Vinogradov & Teis in 1941 (191), by Yoshida *et al.* in 1942 (193), by Dole & Jenks in 1944 (192), and again by Vinogradov and Teis in 1947 (194). Generally only the work of Dole & Jenks has been cited in this connection. However, there has not appeared an adequately critical comparison of the techniques employed in the several laboratories which might serve as a basis for preference of some results over those of other investigators. It is illuminating to tabulate the various results in terms of the fractions of the photosynthetic oxygen apparently originating from water and carbon dioxide respectively (Table 1). Rejecting the hypothesis that the source of photosynthetic oxygen depends upon whether it is investigated in Moscow, Evanston, or Tokyo, we feel that the above array of experimental results does little to bolster our confidence in the theoretically well-nigh inescapable conclusion that *all* the photosynthetic oxygen should originate from the water.

TABLE I

ORIGIN OF PHOTOSYNTHETIC OXYGEN

Per cent of photosynthetic oxygen from		Reference
H_2O	CO_2	
61	39	191
67	33	193
85	15	192
81	19	194

The situation has a further discouraging complication. It so happens that the calculated equilibrium constant at 20°C. for the reaction, $O^{16}O^{18}{}_{(g)} + H_2O^{16}{}_{(l)} \leftrightarrows O^{16}O^{16}{}_{(g)} + H_2O^{18}{}_{(l)}$, is such as to account for a small concentration of heavy isotope in gaseous oxygen. If the photosynthetic oxygen were to equilibrate with the cellular water before being evolved, then its isotopic composition would indicate (perhaps incorrectly) that some had originated from carbon dioxide. Dole & Jenks (192) suggested very tentatively that this might be the explanation for their finding that about one oxygen atom in seven apparently came from carbon dioxide. Since the isotope exchange reaction in question is extremely slow, Kamen & Barker (190) and Kamen (195) seriously considered the possibility that it might be accelerated by living cells. More recently Bentley (183) virtually accepted it as probable. It is remarkable to find in one and the same paper (192) a set of results which are cited as definitive confirmation of water as the sole source of photosynthetic oxygen and also a suggestion of biologically catalyzed isotope exchange be-

tween water and molecular oxygen which, if true, completely invalidates the method. We are forced to conclude that the only kind of result which, in principle could not be seriously questioned on the basis of the effect of exchange reactions, would have been the finding that all or nearly all the photosynthetic oxygen arose from carbon dioxide! Probably investigators of this problem would have defended such a skeptical attitude all along except for the fact that there are compelling indirect theoretical considerations which *a priori* predict the type of result first obtained by Ruben *et al.* The present reviewers do not wish to suggest an alternative concept but want only to emphasize that the experimental evidence from tracer oxygen experiments in several different laboratories not only shows wide disagreement but, on several counts, it is simply inadequate to support or deny the widely accepted theory of water as the sole source of photosynthetic oxygen.

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BIOCHEMISTRY OF ANTIBIOTICS[1]

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Introduction

It seems that it is becoming more and more difficult to determine what material, facts, and hypotheses belong differentially in such fields as organic chemistry, biochemistry, biology, and microbiology, and perhaps we should include also investigative clinical medicine. Needless to say, however, there is basic chemistry in all these areas, but there are, and must be, differences of viewpoint, of interpretation, and of emphasis. As a chemical agent the alkaloid dichroine from Hydrangea becomes the antimalarial antibiotic dichroin. As a representative complex organic molecule, dichroine occupies in *Chemical Abstracts* about 27 pages of space; as an antibiotic it fits into about one page among these Reviews. No doubt the fields of study, as referred to above, may take little or no note of this antibiotic unless malaria looms again and a powerful antimalarial is needed in emergencies. We think this is all as it should be, so that in preparing these Reviews we admit picking and choosing, according to our judgments of timeliness, interest, and preferences, among the all too numerous papers (for inclusion), and we assume justification for the procedure, because of the limitations of space necessarily imposed.

For material omitted, apologies may be made and more particularly to those who might like to know more regarding current clinical aspects. Unfortunately, the clinical literature in its antibiotic relations is perhaps less available to chemists and other nonmedical academic and industrial scientists. However, there are many publications of the year which serve as reviews in the field of the clinical applications. Reference may be made in particular to publications by Welch & Lewis (277), Florey (92), Dowling & Lepper (79), and many special books and monographs, some of which are prepared as compilations of medical contributions and published by industrial firms producing antibiotics. More general references are new books by Karel & Roach (155), and the Council of the Pharmaceutical Society of Great Britain (64).

We feel that the trend today is very strongly in favor of a very liberal interpretation of the term antibiotic. In any event, emphasis on a purely "microbiological" source is confusing. Aside from the fact that every living thing is, in cellular detail, microbiological, there are, for example, certain large groups of the fungi with extensive mycelial growth and some with sporophores weighing several pounds individually. Seed plants furnish, we may assert, many antibiotic substances besides quinine.

[1] The survey of the literature pertaining to this review was concluded in November, 1952.

Antibiotics from Actinomycetes

The broad-spectrum antibiotics aureomycin, terramycin, and chloramphenicol, derived from species of *Streptomyces*, are now so well established as to require little further comment on their clinical value. They are discussed first followed by streptomycin which, though not ideal, remains the more important antibiotic in combating tuberculosis. Other antibiotics from actinomycetes are discussed in alphabetical order.

Aureomycin and terramycin structures.—The structural similarity between aureomycin and terramycin has been suspected on the basis of biological and preliminary chemical information. Further evidence of their close similarity was provided by crystallographic studies by Dunitz & Robertson (84) and Pepinsky & Watanabe (214) which showed that the two antibiotics were isomorphous. With the recent announcements of the structures of terramycin (I, p. 462) by Hochstein *et al.* (136) and aureomycin (II, p. 462) by Waller *et al.* (271), the full extent of the similarity is revealed. In view of the assigned structures for these two antibiotics it is perhaps more surprising that there are so many differences in biological and chemical properties rather than that there are similarities.

The determinations of the structures for these two antibiotics are undoubtedly the most important chemical contributions to the study of antibiotics in the past year, climaxing an estimated 100 man-years of effort, but the description is here restricted to the briefest possible comment. A series of products of degradation of terramycin have been reported by Pasternack *et al.* (209, 210), Hochstein *et al.* (134 to 137), and Kuhn & Dury (164). Some of these products were briefly reviewed by Work (286). The structure of these compounds was established and in some cases verified by synthesis (134). Another and in most cases considerably different series of degradation products has been reported from aureomycin by Hutchings *et al.* (140, 141) and Waller *et al.* (271 to 274). The structures of these compounds were also announced, and some of them have been synthesized by Kushner *et al.* (167). The intermediate compounds formed during the degradation of both of these antibiotics were, in many cases, the result of loss of the carboxamide and the dimethylamine moieties and rearrangement of the naphthacene nucleus due to the labile repeating β-diketone structure. At the time of writing the two structures are considered proven with the possible exception of the relative positions of the 4a hydroxyl and the dimethylamine group (136, 271) and, of course, the stereochemical configurations.

The arrival at these two very similar structures by different indications and lines of reasoning is indirect evidence for their respective correctness. More direct confirmation has also been obtained by the application of degradation reactions of terramycin to aureomycin (250).

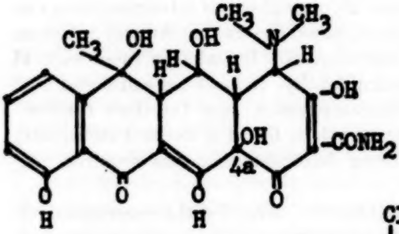
Chloramphenicol.—Several reports have appeared in the past year which link chloramphenicol administration to the production of certain disorders of the blood, the most serious of which is aplastic anemia. In a

survey article Lewis *et al.* (178) have summarized reported cases and conclude that beyond a reasonable doubt chloramphenicol administration can cause, in certain susceptible individuals, blood dyscrasias. Among 539 cases reviewed, 55 had received only chloramphenicol. Included in these were 44 cases of aplastic anemia, 23 terminating fatally. It has been estimated that 8,000,000 patients have received chloramphenicol, and therefore the incidence of such myelotoxicity must be very low, but it is deemed sufficiently serious that the U. S. Food and Drug Administration has required precautionary labeling of the product.

The structure of chloramphenicol [D_G = (—) *threo*-2-dichloroacetamido-1-*p*-nitrophenyl-1,3-propanediol] has been confirmed by crystallographic studies by Dunitz (83) who also studied bromamphenicol. The synthesis and antibacterial evaluation of compounds structurally related to chloramphenicol have continued (9, 35, 63, 73, 87, 96, 130, 194 to 197, 226, 235). Billet & Marnay (35) and Moersch *et al.* (194) have continued studies on the *β*-*p*-nitrophenylserine series of analogues while Molho & Molho-Lacroix (195, 196, 197) have compared these with some *β*-thienylalanine compounds related to chloramphenicol. Herman & Kreuchunas (130) have also prepared chloramphenicol analogues having the phenyl group replaced by a thienyl group. Ashley & Davis (9) have reported the preparation of a series of simpler analogues of chloramphenicol and its molecular fragments. Funke & Kornmann (96) have reported *p*-amino and acylated *p*-amino analogues. Eiter & Sackl (87) have prepared *dl*-desoxychloramphenicol using fermentative reduction with yeast as one of the steps in the synthesis. Further work on the *p*-methoxy and *p*-phenoxy analogues has been reported by Rebstock & Pfeiffer (226). Collins *et al.* (63) have studied the preparation and properties of a series of analogues of chloramphenicol with substituted methyl groups on the aliphatic side chain, and Santarato (235) has studied the antibacterial activity of a series of analogues with differing acyl groups.

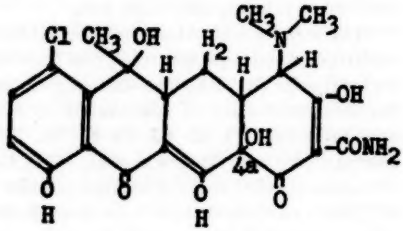
Summarized, this work indicates that changes in the aliphatic portion of the chloramphenicol molecule generally have resulted in drastic reduction or loss of activity whereas changes in the aromatic portion leaves some activity in many cases. Analogues that exhibit inhibitory activity have often appeared to differ qualitatively (antibacterial spectrum, method of action) as well as quantitatively from chloramphenicol. Notable are the *p*-methylsulfonyl and *p*-methylsulfide compounds reported by Cutler *et al.* (73) and other compounds with small *para* substituents, some of which are reported to be quite active antibacterials. Such compounds have taken on a particular interest in view of the reported effect of chloramphenicol on the bone marrow which might be a result of the presence of an aromatic nitro group in the molecule.

An interesting derivative, chloramphenicol palmitate, has been described by Glazko *et al.* (97). Although itself inactive, the compound is enzymatically hydrolysed in the intestine and is slowly absorbed in active form with resultant lower but more prolonged inhibitory blood levels. It offers advantage



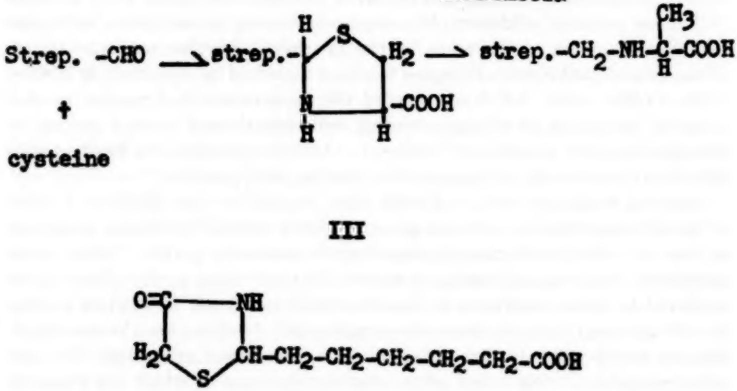
I

TERRAMYCIN



II

AUREOMYCIN



III

IV

ACTITHIAZIC ACID (MYCOBACIDIN)

in that it is tasteless (chloramphenicol is bitter), and it appears to be even less toxic. It has been used to treat pediatric cases with success by Ross *et al.* (231) and Larkin (168).

Streptomycin and related antibiotics.—An interesting and apparently highly specific method of purifying streptomycin has been described by Rhodehamel *et al.* (227). It involves extraction of streptomycin from salt solutions with alkyl or aralkyl amines. The method is believed to operate via a Schiff's base or alcohol-ammoniate intermediate since dihydrostreptomycin is not extracted, and secondary and tertiary amines do not extract streptomycin.

Winsten *et al.* (284) have patented a method of preparation of derivatives of streptomycin which are stated to be active against gram-positive and gram-negative organisms. The products, called streptomycyl amines, can be produced by reductive desulfurization of the product of mercaptoamine inactivation of streptomycin using Raney nickel. This process may be represented by formula III (p. 462).

The characteristics of *Streptomyces griseocarneus* which produces hydroxystreptomycin have been studied by Benedict *et al.* (31). The production of the antibiotic was also outlined. The antibacterial activity of hydroxystreptomycin, streptomycin, and the respective dihydro compounds is very similar. No mannosido-hydroxystreptomycin was produced, and this may indicate a different route of synthesis as compared with streptomycin. The previously postulated structure for the pyrone produced by the action of alkali on hydroxystreptomycin has been verified by Stodola (251) through its conversion to maltol.

Also in the past year Fried and associates (94, 95) have published detailed proof of the linkage of the mannose moiety to the 4-position of the N-methyl-L-glucosamine moiety of mannosidostreptomycin. Karow *et al.* (156) have produced C^{14} labeled streptomycin by biosynthesis from uniformly labeled glucose or starch. Perlman & Wagman (215) have found that it is possible to substitute lipids for glucose in streptomycin fermentations with maintenance of streptomycin yields. Chemical methods of determination or color reactions of streptomycin and its relatives have been reported by Vail & Bricker (264), Monastero (198), and Halliday (118).

Achromycin.—A basic antibiotic, achromycin, has been prepared in crystalline form from *Streptomyces albo-niger*, a new species [Porter *et al.* (216)]. The base melted at 175.5–177°C. and contained 56.55 per cent carbon, 6.35 per cent hydrogen, and 20.88 per cent nitrogen with no halogen, sulfur, phosphorus, or metals present. The intravenous toxicity to mice was of the order of LD_{50} 350 mg./kg. Achromycin was active against both gram-positive and gram-negative bacteria *in vitro* and *Trypanosoma equiperdum* in mice.

Actinomycins.—A species of *Micromonospora* resembling *M. globosa* has been shown to produce an antibiotic which is evidently an actinomycin [Fisher *et al.* (89)]. The crystalline compound, m.p. 251–252°C., contained 60.06 per cent carbon, 6.99 per cent hydrogen, and 12.62 per cent nitrogen.

Absorption maxima were exhibited at 239 and 440 mμ. Paper chromatography of an acid hydrolysate revealed the presence of proline, sarcosine, threonine, valine, and N-methylvaline. The crystalline product inhibited *Bacillus subtilis*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis* 607 at 0.2 0.31, and 6.3 μg./ml. respectively; *Escherichia coli*, *Proteus vulgaris*, *Eberthella typhosa*, and others were not inhibited at 50 μg./ml.

Brockman & Pfennig (50) have reported the separation of actinomycin C by countercurrent distribution into three components, C_1, C_2, and C_3. These are shown to differ in their amino acid composition by paper chromatography of acid hydrolysates.

Studies on the chromophoric grouping of actinomycin B have been published by Johnson *et al.* (148). They have revised the previously published molecular formula of actinomycin B to $C_{61}H_{88}O_{16}N_{12}$ in the light of an improved molecular weight of 1240 ± 20 obtained by quantitative hydrogenation. This formula would require nine moles of amino acid in the peptide portion rather than five as previously reported. At least part of the additional amino acid content is probably D-valine. The peptide-free quinone, actinomycinol B, was produced by hydrolysis with barium hydroxide. This product is a modification of the chromophoric group of actinomycin. It can be represented by the formula $C_{12}H_5ON(2CO)(2CH_3)(2OH)$.

Actithiazic acid (*Mycobacidin*).—Three different groups have reported the isolation of an antibiotic with high specific activity *in vitro* against the mycobacteria. At least one of the organisms producing the antibiotic is apparently a new species and has been described by Grundy *et al.* (112) who propose the name *Streptomyces virginiae*. Sobin (249), Tejera *et al.* (258), and Schenck & DeRose (237) agree that the compound has the molecular formula $C_9H_{15}O_3NS$, m.p. 138–141°C., and is a monobasic acid with an optical rotation of the order of $[\alpha]_D^{25} - 54$ to $60°$($c = 1$, methanol or ethanol). The structure of the compound has been determined to be 4-thiazolidone-2-caproic acid (IV, p. 462) by McLamore *et al.* (188) and Schenck & DeRose (237) and verified by synthesis by McLamore *et al.* (188) and Clark & Schenck (62). The synthetic racemate has about one-half the activity of the natural (*levo*) form. Other derivatives and homologues have been synthesized (62, 188). The unsubstituted amide and esters of lower alcohols were found to be about twice as active *in vitro* but more toxic than the free acid (62). The optimum length of the side chain for microbiological activity was five methylene groups (62). The toxicity of the compound is very low as reported by Tejera *et al.* (258) and Hwang (142). It was not, however, active *in vivo* according to Hwang (142) when tested against experimental tuberculosis in the mouse.

The lack of activity *in vivo* is probably due to the presence of biotin (142), since Grundy *et al.* (112) have shown that the presence of biotin eliminates the effect of ordinarily inhibitory levels *in vitro*. Mycobacteria do not ordinarily require biotin, and it is believed that this antibiotic acts by interference with the synthesis of biotin (112).

It is a significant commentary on the pace prevailing in the field of antibiotics when an antibiotic is isolated by three separate groups, its structure determined and verified by synthesis, its microbiological and pharmacological properties outlined, and at least a broad hint as to its mode of action published in only one year.

Antimycin.—Schneider *et al.* (239) have resolved preparations of this antifungal antibiotic into active fractions A and B by paper chromatography. The A fraction represented 85 to 90 per cent of the total activity. Antimycin A ($C_{28}H_{40}O_9N_2$) was obtained as a crystalline product with constant melting point upon recrystallization; however, this material was still impure and has been purified by partition chromatography. Analyses and spectra of the purified product were essentially the same as those of the original crystalline preparation. Bumpus *et al.* (52) have degraded antimycin A by gentle saponification to a $C_{11}H_{14}O_5N_2$ phenol, a fatty acid probably L (+)-methylethyl acetic acid, and an oily neutral fraction. More vigorous saponification gave a mixture of four acids separable by partition chromatography.

Ascosin.—Hickey *et al.* (132) have reported an antifungal antibiotic which they have named ascosin. It is produced by an organism which they characterized as a new species and named *Streptomyces canescus*. The active material occurs in both the mycelium and the culture filtrate and has not yet been isolated in pure form. Tests of the material appear to differentiate it from several other antifungal antibiotics. The material is shown to be active *in vitro* against yeasts and some filamentous fungi, but not against bacteria. With intravenous injection the LD_{50} with mice was about 12.5 mg./kg. and nerve cell degeneration was seen.

Catenulin.—Another member of the neomycin group of antibiotics has been isolated from a *Streptomyces* species not considered *S. fradiae*, by Davisson *et al.* (75). The analyses of salts and other properties are similar to members of the neomycin complex especially neomycin B. A product of the acid hydrolysis of catenulin was tentatively identified as neamine. Catenulin was differentiated from several antibiotics including neomycins A and B by paper chromatography. Cumulative toxicity and neurotoxicity were observed during prolonged administration to cats.

Carbomycin (*Magnamycin*).—Tanner *et al.* (257) have published a brief report describing some of the properties of carbomycin. It is a basic antibiotic isolated from submerged cultures of a strain of *Streptomyces halstedii*. The crystalline base gave a neutral equivalent about 860, $[\alpha]_D^{25} -58.6°$ (chloroform), and a single absorption maximum in the ultra-violet region at 240 mμ. It contained 59.0 per cent carbon, 8.1 per cent hydrogen, and 1.7 per cent nitrogen.

Carbomycin was active against gram-positive bacteria, *Rickettsia*, and large viruses. Resistance is stated to be developed in a slow, step-wise manner, and no cross-resistance with penicillin, aureomycin, chloramphenicol, terramycin, bacitracin, polymyxin B, or streptomycin was found. Carbomycin was absorbed when administered orally and was of relatively

low toxicity (LD_{50} 550 mg. hydrochloride/kg., mice, intravenously).

Preliminary clinical trials are stated to be promising, and this antibiotic, together with erythromycin which it seems to resemble, may soon be available for the treatment of infections resistant to other antibiotics.

Erythromycin (*Ilotycin*).—McGuire *et al.* (187) have reported the isolation and partial characterization of a new antibiotic, erythromycin, from a strain of *Streptomyces erythreus* (Waksman) isolated from Philippine soil. The antibiotic could be extracted with solvents from the culture filtrate at alkaline pH and was isolated in the form of white crystals. Erythromycin has a single basic group (pK 8.8), a molecular weight of about 725 and an empirical formula of the order of $C_{34-36}H_{60-65}NO_{11-14}$. The ultra-violet spectrum showed a single broad peak at 280 mμ.

The properties announced by McGuire *et al.* (187) have been confirmed and extended by studies reported by Heilman *et al.* (128), Haight & Finland (113 to 116) and Anderson *et al.* (5). Erythromycin was found to be active *in vitro* primarily against gram-positive organisms, but some gram-negative organisms, such as *Hemophilus*, *Brucella*, and *Neisseria*, were also sensitive. Some activity was also reported against *Rickettsia* and large viruses *in ovo*. Protective activity *in vivo* was reported and acute and subacute toxicity found to be low. Details of limited clinical treatment of patients with various illnesses are reported (113, 128) with generally favorable results.

Gastrointestinal irritation did occur in some of these cases especially with high dose levels (113, 128). A maximum blood level was reached in about one to two hours following oral administration. It is excreted, at least partly in active form, via the urine and the bile, and demonstrable blood levels persist about six hours following a single oral dose (5, 114, 128). Orally administered erythromycin diffused readily into the body tissues and fluids tested with the possible exception of the cerebrospinal fluid (5, 128).

Erythromycin is bactericidal or bacteriostatic depending on the organism and the concentration (116, 128). It acts only on multiplying bacteria (116). Cultures grown in the presence of the drug were not found to produce inhibitors of the activity (113, 115), but resistance does develop both *in vitro* and clinically (113, 128). No cross resistance with other antibiotics (including aureomycin, bacitracin, chloramphenicol, neomycin, penicillin, polymyxin, streptomycin, and terramycin) has been demonstrated (115, 128, 187). If resistance is not too rapidly produced, this antibiotic should find a place in therapy particularly in the treatment of infections caused by gram-positive organisms resistant to penicillin or occurring in patients sensitive to penicillin.

Helixin.—An apparently new species of *Streptomyces* has been found by Leben *et al.* (172) to produce an antifungal antibiotic. This antibiotic, named helixin, was assayed using *Glomerella cingulata* on agar plates. The activity could be isolated from the culture fluid by precipitation at pH 3 and extraction of the precipitate with ethanol. By suitable solvent manipula-

tion helixin was obtained as a red gum which inhibited completely all fungi studied in the range 0.47 to 15 mg./cc. It also inhibited some bacteria. Smeby *et al.* (246) have purified the crude helixin further by partition chromatography. From paper chromatograms it appears that crude helixin contains four active components which are designated A, B, C, and D. Helixins A, B, and C have been obtained essentially free of each other by solvent fractionation. Helixin B and one of the active components of Endomycin (98) are very similar and quite possibly identical as revealed by comparative paper chromatograms.

Mycomycin.—Mycomycin was first announced by Johnson & Burdon (150) who produced solvent extracts of a culture later identified as *Nocardia acidophilus* which were highly active against the bacillus of human tuberculosis and nontoxic for mice. Although hampered by extreme instability of the substance, Celmer & Solomons (58) have isolated mycomycin in crystalline form by working at low temperatures and under an inert atmosphere. Complete retention of activity was obtained only at less than −40°C., and the half-life of the crystalline solid under nitrogen at 27°C. was only 3 hr. The crystalline antibiotic is an optically active, $C_{13}H_{10}O_2$, monobasic acid which explodes at 75°C. (57, 58). It absorbs eight moles of hydrogen to form *n*-tridecanoic acid. From a study of derivatives, properties, and spectra of mycomycin and an alkali rearranged product, isomycomycin, the respective structures 3,5,7,8,-tridecatetraene-10, 12-diynoic acid (V) and 3,5-trideca-diene-7,9,11-triynoic acid (VI) have been assigned to these two compounds by Celmer & Solomons (57, 58, 59).

V $HC{\equiv}C{-}C{\equiv}C{-}CH{=}C{=}CH{-}CH{=}CH{-}CH{=}CH{-}CH_2{-}COOH$

VI $H_3C{-}C{\equiv}C{-}C{\equiv}C{-}C{\equiv}C{-}CH{=}CH{-}CH{=}CH{-}CH_2COOH$

In view of the announced structure, it is not surprising that mycomycin is unstable; however, an explosive polyacetylenic antibiotic whose optical activity is due to an unsymmetrical allenic structure is hardly commonplace.

Following this well nigh heroic chemical work it must have been particularly disappointing to find that the crystalline mycomycin was inactive *in vivo* although active *in vitro* against several microorganisms including both streptomycin-sensitive and resistant tubercle bacilli and certain pathogenic fungi (58). Jenkins (147) reports that despite *in vivo* activity against experimental tuberculosis shown in earlier tests with crude preparations, the pure mycomycin was inactive. It was felt that some additional factor must have been lost during purification especially since it was possible to demonstrate blood levels in the guinea pig.

Neomycins.—The neomycin complex as isolated from *Streptomyces fradiae* cultures consists of at least three forms. Leach & Teeters (170) and Dutcher & Donin (85) have reported the identity of neomycin A with a degradation product of the other members of the complex, neamine. Neamine has been reported to conform to the formula $C_6H_{12-14}O_3N_2$, and yet the methanolysis product from neomycins B and C which is now shown to be

the same as neamine was reported as $C_9H_{19}O_6N_3$. This discrepancy results from the experimental molecular weights, and it seems probable that neamine will be found to be C_9 or C_{12} since it has been shown to form a known $C_6H_{14}O_3N_2$ compound (1,3-diamino-4,5,6-trihydroxycyclohexane) upon boiling with hydrobromic acid solution.

While having a reasonably low acute toxicity the neomycin complex is nephrotoxic and ototoxic, and therefore its parenteral use has been largely in serious cases resistant to other agencies. Nesbit *et al.* (205) used a low but clinically effective dosage with considerable success in combating urinary tract infections refractory to from one to five other chemotherapeutants. Since neomycin is rapidly bactericidal and poorly absorbed following oral dosage, its use as an intestinal antiseptic has been studied by Poth *et al.* (217) and Schweinburg *et al.* (241). With the exception of yeasts and *Clostridia* most of the fecal flora are considerably reduced or eliminated, and the treatment has been used with favorable results as a preparation for intestinal surgery. The acute toxicity and protective effect with experimental tuberculosis was found to be about the same with neomycins B or C by Hamre *et al.* (119). Iverson & Waksman (143) have studied the development of resistance to neomycin by *E. coli.*

Nigericin.—Harned *et al.* (121) have enlarged upon their earlier report (122) on nigericin, an antibiotic produced by an unidentified species of *Streptomyces* from Nigerian soil. Nigericin can be isolated from culture filtrates by acidic precipitation, charcoal adsorption, or solvent extraction. The free acid was not obtained in crystalline form, but the crystalline sodium salt conformed to the molecular formula $C_{39}H_{68}O_{11}Na$. Gram positive organisms are most sensitive to the antibiotic, but activity was shown against certain representative gram-negative forms as well as by species of *Mycobacterium*, *Candida*, and *Trichophyton*. The LD_{50} in mice was about 2.5 mg./kg. intraperitoneally.

Nitrosporin.—Umezawa & Takeuchi (261, 262) have isolated an apparently new species which they have named *Streptomyces nitrosporeus*. This organism produced a basic antibiotic which was extractable into solvents. Following chromatography on alumina, the crystalline nitrosporin obtained melted at 115–120°C. (decomp.) and corresponded approximately to the molecular formula $C_{20}H_{26}N_2O_6$. This substance gave an estimated LD_{50} of 16 mg./kg. when injected intravenously into mice. It inhibits gram-positive bacteria primarily and within this group has sharp differences in potency, e.g., the minimum inhibitory concentration for group A *Streptococcus hemolyticus* is about 0.3 μg./cc. but for group C it is greater than 100 μg./cc.

Nocardianin.—Bick *et al.* (34) have isolated nocardianin by ether extraction and chromatography on alumina as red crystals, m.p. 228–235°C. (decomp.). The antibiotic is active against gram-positive organisms only and is rather unstable especially in solution. It is produced by a species of *Nocardia* in surface culture. The molecular formula is of the order of $C_{64}H_{100}O_{15}N_{18}$.

Streptolins.—The crude streptolin isolated from *Streptomyces* S-11 cultures is shown to contain active components A and B by paper chromatography [Larson *et al.* (169)]. By fractional crystallization of the helianthate streptolin A was obtained free of streptolin B. The analyses of the sulfate and helianthate are stated to be in agreement with the formula $C_{24}H_{46}O_{11}N_7$ for streptolin A free base. The mother liquors from the helianthate crystallization yielded a sulfate which consisted primarily of streptolin B and was more active on a weight basis than the sulfate of streptolin A. Smissman *et al.* (247) found that streptolin sulfate (A+B) apparently contained no alkoxyl, alkimide, or terminal methyl groups. An acid hydrolysate was fractionated on an ion-exchange resin column and the presence of ammonia, a reducing fraction probably an amino sugar, a $C_4H_{8-10}O_3N_2$ base, and a six carbon diamino-carboxylic acid was indicated. The latter compound has been shown to be β,ϵ-diaminocaproic acid by van Tamelen *et al.* (265)

Streptothricin.—Continuing their studies upon the degradation of streptothricin, Carter *et al.* (56) have announced determination of the structure of the lysine isomer found in acid hydrolysates of streptothricin. The name β-lysine is proposed for the compound which is shown to be β,ϵ-diamino-*n*-caproic acid by comparison with the synthesized compound. The same amino acid has been produced from viomycin and streptolin (123, 265).

Viomycin.—Haskell *et al.* (123) have reported in detail the information given in last year's review. It is understood that the lysine isomer from acid hydrolysates of viomycin is the same as that identified by Carter *et al.* (56) from streptothricin and van Tamelen (265) from streptolin.

The use of viomycin clinically, especially against tuberculosis, is apparently still under trial. As far as is yet known it is not superior to streptomycin except perhaps in cases resistant to streptomycin.

Other antibiotics from Actinomycetes.—Three new crystalline antibiotics from species of *Streptomyces* have been reported by Berger *et al.* (32). The compounds are colorless, optically active acids with approximate molecular formulae $C_{46-47}H_{80-82}O_{13}$, $C_{25}H_{40}O_7$, and $C_{34}H_{62}O_8$. They are all active *in vitro* against certain gram-positive bacteria and *Mycobacteria.* They are, however, rather toxic and are inactive *in vivo* at tolerated levels against bacteria and protozoans.

Charney and associates (46, 60, 61, 181) have announced four apparently new antibiotics. Thioaurin, $C_{14}H_{12}O_4N_4S_4$, m.p. 179–181°C., is a bright orange-yellow antibiotic active against gram-positive and gram-negative bacteria but relatively inactive against molds and yeasts and having an intravenous toxicity of LD_{50} 16 mg./kg. for mice (46). Cardicin (181) from species of *Nocardia* has antifungal, antibacterial, and antiviral activity but is toxic. Rhodocidin (60) is a violet pigment from an unidentified species of "actinomyces." It is active against bacteria including *Staphylococcus aureus*, *Proteus vulgaris*, and *Mycobacterium* H37RV. It shows protective activity in experimental *Streptococcus* infections. Pleocidin (61) is a streptothricin-like antibiotic with an intraperitoneal LD_{50} of about 3 mg./kg. for mice.

Cosar *et al.* (65) have isolated a crystalline hydrochloride $C_{10}H_{21}O_3N_5 \cdot HCl$ of a basic antibiotic. It decomposes at 205–210°C. It shows *in vitro* activity against gram-positive, gram-negative, and acid-fast organisms. The *in vivo* activity against bacteria appears to be weak, but the trypanocidal activity in the mouse was strong. The LD_{50} for the mouse was of the order of 250 mg./kg. subcutaneously.

Ehrlichin, an antiviral antibiotic preparation, was described by Groupé *et al.* (103). Phagolessin A58 has activity against bacteriophages and some antibacterial action. It is toxic to mice, but might eliminate phage from lysogenic strains of "actinomyces" and bacteria [Asheshov *et al.* (8)]. Another antiviral substance, Abikoviromycin has been reported by Umezawa *et al.* (263). It is also quite toxic and is unstable.

Raubitscheck *et al.* (225) have reported work on an antifungal antibiotic very similar to fungicidin. Hidy & Hickey (133) have described a chromatographic and a solvent extraction-precipitation method for the isolation of fradicin. The protozoicidal, fungicidal, and bactericidal properties of thiolutin have been described by Seneca *et al.* (243), and its favorable effect on tinea capitis has been reported by Franks (93). Degradation studies on xanthomycin A ($C_{31}H_{36}N_4O_8 \cdot 3HCl$) by Rao & Petersen (224) have shown the presence of a quinoid structure with one methylimide and four methoxyl groups. Acid hydrolysis produced ethanolamine, methylamine, and ammonia in the molar ratio of 2:1:1. In a continuation of their previous work Brockman *et al.* (49) have given details of further characterization of picromycin ($C_{25}H_{43}O_7N$). Two ester or lactone groups are indicated. Rimocidin from *Streptomyces rimosus* was reported last year by Davisson *et al.* (76). It has an intravenous LD_{50} of about 30 mg./kg. for mice and is protozoicidal [Seneca *et al.* (244)] as well as antifungal. Benedict *et al.* (30) have recently reported a new polypeptide antibiotic, cinnamycin, from *Streptomyces cinnamoneus*. It appears to contain aspartic acid, arginine, glutamic acid, proline, phenylalanine, valine, lanthionine, and the $C_7H_{14}N_2O_4S$ amino acid isolated from subtilin.

Fungi Imperfecti

Penicillin.—The production of penicillin salts with many organic bases, especially those resulting in relatively stable and insoluble salts which might have advantages as repository forms etc., continues apace particularly as evidenced in the patent literature. It is felt, however, that no present useful purpose would be served in reviewing these. The procaine salt of penicillin G remains the standard repository preparation. One of the more promising new salts of this type was reported by Elias *et al.* (88), Szabo *et al.* (254) and Seifter *et al.* (242). They have reported the preparation and properties of N,N′-dibenzylethylenediamine dipenicillin. The compound compared favorably with procaine penicillin in several respects and unusually prolonged blood levels were produced following a single injection of an aqueous suspension. The oral administration of this compound in com-

bating bacterial infections has been favorably reported by Lepper *et al.* (177).

Further work on the hydriodide of β-diethylaminoethyl ester of benzylpenicillin was reported by Hallas-Moller *et al.* (117), Flippin *et al.* (90), and Rhodes *et al.* (228) who confirm earlier reports that the compound concentrates in the lungs. It appears that this ester is suitable for repository administration especially in the case of penicillin-sensitive lung infections. The concentration of penicillin produced in the tissues is lower than with procaine penicillin (90, 117). Since esters of penicillin are inactive until hydrolyzed, Dinsmore & Bailey (78) and Keller (157) have devised and applied methods for the study of the rate of hydrolysis of this ester based on the differences in partition coefficients in various solvent systems between it and free penicillin.

Mortimer & Johnston (199) investigated the structural requirements for efficient penicillin precursor action in a series of organic acids in the light of modern techniques including paper chromatography. It was found that the acid must not be substituted in the α-position if the corresponding penicillin is to be formed. The rate of metabolism of the acid bore an inverse relation to precursor efficiency. Forms of the acids tending to resist β-oxidation were better as precursors. If an acid that is metabolized rapidly is added in frequent small increments during the fermentation, precursor efficiency is enhanced. Tabenkin *et al.* (256) found that higher alkyl esters of phenylacetic acid were precursors for and stimulated production of benzylpenicillin to about the same extent as β-phenylethylamine. The lower alkyl esters of phenylacetic acid were toxic to the mold. Rolinson (230) has studied the respiration of *Penicillium chrysogenum* in penicillin production, and Johnson (151) has reported recent developments in penicillin fermentations.

The clinical use of penicillin has been comprehensively reviewed by Florey (92). Triantaphyllopoulos & Waisbren (260) have studied the controversial effect of penicillin upon blood coagulation in more detail than had been done heretofore, and no effect was shown with crystalline penicillin *in vivo* or *in vitro*. They suggest that many of the earlier reports to the contrary may have resulted from impurities present in the penicillin preparation used. Juch (154) has reported that very high levels (35,000 u/ml. or more) will inhibit blood coagulation *in vitro*.

Wachsmuth (268) has reported a series of reactions of penicillin, some of which may be adaptable to qualitative or quantitative estimation, and a series of papers have suggested improvements in analytical methods for penicillin (29, 47, 232, 267).

Alternaric acid.—Further work has been done on alternaric acid, $C_{21}H_{30}O_8$, m.p. 138°C., crystalline, and optically inactive. It is an unsaturated dibasic acid produced in suitable liquid media by *Alternaria solani*. It is highly active as an antifungal agent, but the antibacterial activity is negligible. Grove (106) has determined from infrared and ultraviolet absorption spectra, in part, that one of the acidic functions is attributable to a β-dicarbonyl

grouping. Alternaric acid is phytotoxic on leaves of potato, and the lesions incited by it are similar to those incited by the parasitic fungus in its growth on the host. This phytotoxicity probably eliminates this antibiotic as a possible growing-crop protectant fungicide, though it might find application in seed, tuber, and bulb treatment when there may be seed transmission of disease. Pound & Stahmann (219) observed that a substance derived from filtrates of *A. solani*, and probably alternaric acid produced epinasty, wilting, and necrosis of tomato cuttings at dilutions as great as 1 to 50 million.

Fumagillin.—This is an antibiotic from *Aspergillus fumigatus* with relatively little antibacterial and antifungal activity. On the other hand, Killough *et al.* (158) find marked amebicidal potency, noting activity against at least seven protozoan parasites, the more sensitive being *Endamoeba histolytica*. Anderson *et al.* (4) would seem to consider it most promising as a direct-acting amebicide. However, in the cases cited they indicate that at a total dosage of 100 mg. over 12 1/2 days nine out of ten cyst carriers remained clear during three months; but a 50 mg. total dosage cleared only four of ten patients. Further work is suggested. There appears to be no report of analytical data. When the properties of this antibiotic were first studied by Hanson & Eble (120) antiphage activity seemed to be indicated, but utilization of the product in this direction has not made progress. Recently Asheshov *et al.* (7) have shown the identity of fumagillin and phagopedin sigma.

Griseofulvin.—An additional series of important papers is now available on the structural characteristics of griseofulvin ($C_{17}H_{17}O_6Cl$, m.p. 220°C.) [Grove *et al.* (107 to 110); Mulholland (202, 203)]. Structures previously suggested are now shown to be inconsistent with infrared and ultraviolet absorption spectra. By catalytic reduction of griesofulvic acid to two nonlactonic alcohols ($C_{16}H_{19}O_6Cl$ and $C_{16}H_{19}O_5Cl$) and other supporting evidence, griseofulvin is confirmed as the methyl ether of a 1,3-diketone. The significance of the oxidative degradations and the structures of the oxidation products, are fully considered. The structure of the antibiotic is established as in formula VII (p. 476).

Cephalosporin and synnematin.—Several papers on antibiotics from culture filtrates of *Cephalosporium* (or *Tilachlidium*) during somewhat more than a year do not yet present a clear picture, although the contribution by Burton & Abraham (53) established a basis for chemical study. This paper was reviewed last year [Work (286)], and from that it seemed clear that the three substances which had been crystallized, P1, P2, and P4 might offer properties of some further interest, particularly P1, which was reported stable in organic solvents. Gottshall *et al.* (100) have found activity in a related mold, identified as *Tilachlidium*, the active substance being called synnematin. This antibiotic from crude preparations has exhibited what appears to be good activity *in vitro* against both gram-negative and gram-positive bacteria, 33 strains being inhibited at fairly low concentrations, and of the seven fungi tested all were inhibited, though at high concentrations.

A recent paper by Roberts (229) advances almost exclusively certain mycological aspects. Crawford *et al.* (67) studying further cephalosporins P offer important information regarding the fermentation process and solvent relationships. The relationship, if any, of the P antibiotics to synnematin awaits further study.

Albidin.—Albidin is the name given a few years ago [Curtis and Grove (70)] to an antibiotic red pigment derived from culture filtrates of the mold *Penicillium albidum.* It has been shown to be antifungal and antibacterial. A further contribution is made by Curtis *et al.* (71) to the nutritional requirements of the mold in metabolizing this substance, to the stability of the product, and to the minimal concentrations permitting germination of the spores of seventeen species of fungi. Such concentrations range from 0.04 to 3.1 μg/cc.

Fuscin and Ustic acid.—Attention may be called to two substances which have not had a place in recent review papers consulted. These are fuscin and ustic acid. Derivatives and degradation products of fuscin, a metabolite with antibacterial properties from the mold *Oidiodendron fuscum*, have been prepared and described by Birkinshaw *et al.* (37). It is hinted that work on molecular constitution is in progress. Ustic acid is a by-product of the growth of *Aspergillus ustus* on defined media. The filtrates gave an intense purple color with ferric chloride. The substance responsible for this color reaction has been isolated in a pure state and to it Raistrick & Stickings (223) have applied the name, ustic acid. This acid, $C_{11}H_{12}O_7$, m.p. 169 to 170°C., has no optical activity. Derivatives and breakdown products have been prepared, and the substance is shown to be 2,4-dihydroxy-5-methoxybenzoic acid with an unbranched side chain, $-C_3H_5O_2$, assigned to position 6. Of significance and interest is the close structural relationship pointed out between ustic acid and certain other mold products and lichen acids.

Palitantin and frequentin.—In 1936 Birkinshaw & Raistrick (39) described palitantin, $C_{14}H_{22}O_4$, from *Penicillium palitans*, the product being characterized in part as an aldehyde with two hydroxyl groups and probably two double bonds. From recent studies Birkinshaw (36) has prepared derivatives, and particularly has followed quantitatively the oxidation of the substance and of derivatives with periodate. Re-examination of palitantin reveals a low but definite optical activity. An accompanying acidic substance from *P. palitans* proved to be identical with the substance frequentin from *Penicillium frequentans*, and Curtis & Duncanson (69) were able to show that both palitantin and frequentin give dihydropalitantin on reduction with sodium amalgam, indicating that in these two metabolites the carbon skeletons and positions of oxygen atoms are identical.

Cyclopaldic and gladiolic acids.—Birkinshaw *et al.* (41) have isolated cyclopolic and cyclopaldic acids from a strain of *Penicillium cyclopium* and have determined the structures. Smith (248) finds considerable antifungal activity in cyclopaldic acid. Grove (105) and Raistrick & Ross (222) have

reported the chemistry of gladiolic acid in detail and have arrived at a structure for this weakly antibacterial, but strongly antifungal antibiotic. Apparently dihydrogladiolic acid (222) and cyclopolic acid (41) are essentially inactive (248) but may be converted into the respective tautomeric active compounds by periodate treatment. The structural relationships are presented.

Other antibiotics.—Birkinshaw *et al.* (40) have published confirmatory evidence for the structure (previously preferred) for mycophenolic acid. In several of the papers referred to under this and the previous side heading (40, 41, 105) the interrelations of some of these products of *Penicillium* spp. with each other and with metabolic products of seed plants has been discussed.

Mycoin C_3 has been shown to be identical with patulin by Baron *et al.* (25). Darken & Sjolander (74) have made detailed suggestions relating to patulin production in submerged fermentation. Williams (282) has reported further work on helvolic acid, $C_{32}H_{42-44}O_8$.

ANTIBIOTICS FROM BASIDIOMYCETES

Ustilagic acid (*Ustizeain B*).—This material is derived from cultures of the corn smut fungus, *Ustilago zeae.* Thorn & Haskins (259) and Haskins & Thorn (124) have studied some of the properties of this complex. From shake flasks or fermenters with fairly standard media high yields (5 to 23 mg./cc.) have been obtained. For a part of the activity glucolipids are responsible. Activity is apparently not affected by recrystallization but is destroyed by alkaline hydrolysis. The antibacterial spectrum is wider on the side of gram-positive bacteria, and the antifungal spectrum is broad. The material is thermostable and relatively nontoxic to the rat and mouse. Lemieux *et al.* (176) and Lemieux (173) were concerned with procedures for isolation, with degradation products, and evidences of heterogeneity. Further, ustilagic acid was regarded as a mixture of partly acylated di-D-glucosyl derivatives of a dihydroxyhexadecanoic acid, called ustilic acid. Infrared spectra of samples of crystalline and amorphous ustilagic acid are reported. Lemieux & Giguere (175) and Lemieux & Charanduk (174) have continued studies of ustilagic acid and report that 60 to 75 per cent of it is monoacetyl-mono-L-β-hydroxy-*n*-caproylglucoustilic acid and 25 to 30 per cent is the analogous caprylyl derivative.

Polyporenic acid C and ungulinic acid.—The wood-rotting fungus *Polyporus benzoinus* has been cultivated on a vimaltol-marmite liquid medium by Birkinshaw and associates (38) with good yields of acidic and aromatic products. The study of the metabolic products is well advanced in this paper. Anisaldehyde identified as the 2,4-dinitrophenylhydrazone is apparently the main contributor to the odor, and two acids requiring special consideration because of antibiotic properties are a triterpenoid acid which appears to be polyporenic acid C, and ungulinic acid. The first is a dihydroxymonobasic acid with a terminal methylene group ($C_{30}H_{46}O_4$ or $C_{30}H_{48}O_4$,

m.p. 285–290°C.) originally obtained by Cross *et al.* (68) from sporophores of *P. betulinus*. Ungulinic acid, given as probably $C_{22}H_{38}O_6$, m.p. 78°C., is accredited with two carboxyls and one lactonic grouping. Antibacterial activity determinations made by Marcus (184) were by the tube dilution method using growth indication in broth, with four test organisms, including *Staphylococcus aureus*, *Escherichia coli*, *Mycobacterium phlei*, and *M. smegmatis*. Against the two last named, the triterpenoic acid is a powerful agent with complete inhibition at 1 to 1,280,000 and 1 to 640,000 respectively, at 72 hours, but it has only low activity against *S. aureus* and *E. coli*. On the other hand, with these four organisms, ungulinic acid is moderately active against only *S. aureus*.

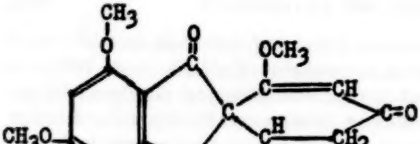
Illudin M and S.—Continuing studies with *Clitocybe illudens*, Anchel and associates (2) have isolated four crystalline substances, two of which, illudin M and illudin S, display antibacterial properties. Illudin M is extracted with chloroform and concentrated *in vacuo* to yield in part the M form. Illudin S is obtained from the culture fluid after chloroform extraction by treatment with Norit A and extraction with ethyl acetate from a concentrate of the aqueous acetone eluate. No further chemical characterization of these illudin substances is indicated.

Antibiotics from Bacteria

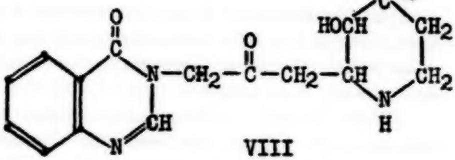
Bacitracin.—Countercurrent distribution studies have convinced Craig *et al.* (66) that commercial bacitracin, a complex peptide, comprises five components, four of which, B, D, E, and F, are regarded as minor constituents. The main peak in the distribution diagram is designated bacitracin A, the chromatographic map of which indicates cystine, ornithine, histidine, lysine, aspartic acid, glutamic acid, phenylalanine, isoleucine, and leucine. Ultraviolet absorption measurements, using an aqueous solution containing 1 mg./cc., displays a weak maximum in the region of 250 to 255 mμ. Bacitracin A is labile to alkali, strong acid, and also to formaldehyde.

Other antibiotics from Bacillus subtilis.—Subtilin has been studied by Carson (55) who finds that its free amino groups are provided two-thirds by the ϵ-amino groups of lysine and one-third by the two constituent sulfur-containing amino acids, lanthionine and an unknown acid $C_5H_8S(NH_2)_2(COOH)_2$. The lysine moieties each have the ϵ-amino group free and the α-amino group bound while the two sulfur amino acids do not occur with both amino groups free. The possible use of subtilin as a preservative in canned foods appears to be still under consideration. Although long-term oral administration appears quite harmless, the relative insensibility of dormant spores, the selective spectrum, and the influence of the size of bacterial population, among other problems, make it doubtful that this or any other single known antibiotic can be safely used in commercial canning operations in the near future [Magdonelle (183); Wilson & De Eds (283); Williams & Fleming (281)].

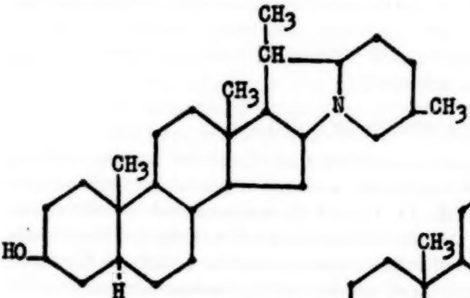
Other antibiotics produced by this ubiquitous organism continue to be



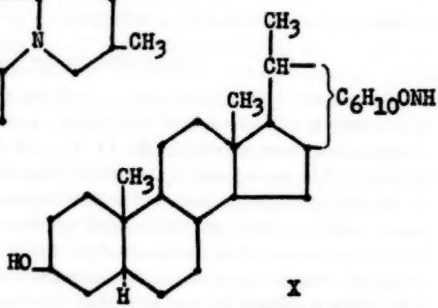
VII
GRISEOFULVIN



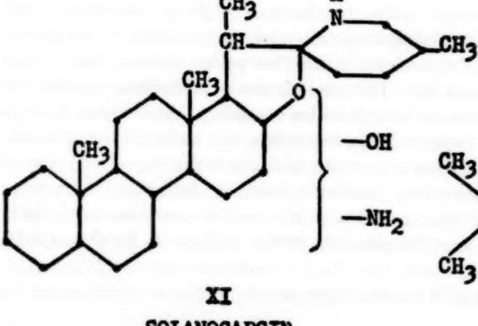
VIII
DICHROIN



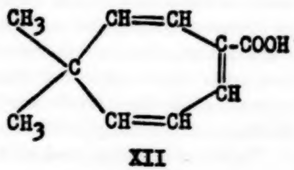
IX
DEMISSIDIN



X
TOMATIDIN



XI
SOLANOCAPSIN



XII
THUJIC ACID

reported. Quinn (221) has reported the production of globicin active against gram-positive and acid-fast bacteria but ineffective against gram-negative organisms. This preparation was differentiated on the basis of its solubility, antibacterial spectrum, etc. from antibiotics previously reported from *B. subtilis* including xanthellin which was announced last year by Wachter *et al.* (269). Delcambe (77) has reported the fractionation of iturin into three active components, and Babad *et al.* (11) have described an antifungal polypeptide from *B. subtilis* which is said to differ from mycosubtilin and fungistatin.

Antibiotics from Pseudomonas.—Wells and associates (278, 279, 280) have determined the structures of the *Pseudomonas aeruginosa* products Pyo Ib, Pyo Ic and Pyo III to be 2-heptyl-4-quinolinol, 2-nonyl-4-quinolinol and 2-(Δ^1-nonenyl)-4-quinolinol respectively. The structures of Pyo II and Pyo IV remain in doubt. Reaction of the Pyo compounds with sodium hypoiodite has been used to produce a series of iodine-substituted derivatives which are much more active against *Staphylococcus aureus* than the parent compounds. The activities against *S. aureus* of both the original and iodinated compounds are greatly reduced in the presence of serum.

Viscosin, a crystalline product from *Pseudomonas viscosa,* is an acidic peptide, m.p. 264–268°C., active against mycobacteria *in vitro* and *in vivo* having some activity against the viruses of bronchitis and influenza A [Groupé *et al.* (104)].

Polymyxin and similar antibiotics.—Brownlee *et al.* (51) have studied the bacteriological and pharmacological properties of polymyxins A, B, and E and to a lesser extent polymyxins C and D. Polymyxins B and E were found to be far less nephrotoxic than polymyxin A (and in preliminary tests also less than C or D). Polymyxin E caused less irritation at the site of injection than polymyxin B. Florestano & Bahler (91) found that varieties B and E were more active than A or D against five dermatophytes and one systemic fungus. Dowling *et al.* (80) have differentiated the closely related circulins A and B from polymyxins A and E by paper chromatography. Short (245) has reported experiments indicating that the protective action of methionine versus the nephrotoxicity of polymyxin A is due to a replenishment of the supply of labile methyl groups.

Hausmann & Craig (125) have shown that a crystalline polypeptin sulfate preparation consists of two basic polypeptides with identical amino acid spectra. The major component (80 per cent) was separated by countercurrent distribution methods and a molecular weight of 1145 and the formula $C_{56}H_{96}O_{13}N_2$ postulated. The amino acid composition corresponds to (L-α, γ-diaminobutyric acid)$_3$, L-threonine, D-valine, D-phenylalanine, (L-leucine)$_2$, and L-isoleucine plus an unknown acid which by difference would be $C_8H_{13}O_2$.

Micrococcin.—The antibiotic micrococcin, derived from cultures of *Micrococcus sp.*, was crystallized by Heatley & Doery (126) and some of its properties described. It is relatively insoluble in water, but in 70 to 80 per cent ethanol the solubility rises to a maximum of about 150 mg./cc. The

molecule has a broad absorption in the ultraviolet, and the ethanolic solution displays purple fluorescence in the near ultraviolet. The substance decomposes at 222–228°C. and has a molecular weight above 2000. The empirical formula is probably about $C_{25}H_{28}O_6N_6S_3$.

Nisin.—Nisin, a relatively insoluble antibiotic, obtained from lactic streptococcal fermentations, described by Mattick & Hirsch in 1944 (186) has had renewed attention from Heatley *et al.* (127) and from Gowans *et al.* (101) among others. An interpretation of the significance of the recent work suggests: (*a*) Nisin appears to be less active against *Mycobacterium tuberculosis* than previously indicated by Bavin *et al.* (28), though it inhibits strongly the growth *in vitro* of many strains of *Streptococcus pyogenes*. (*b*) Conditions may be arranged such that the drug protects against experimental infection of mice with *S. pyogenes* and *Staphylococcus aureus*, but no protection has been demonstrated against infection with a bovine strain of *M. tuberculosis* in rabbits. (*c*) According to Berridge (33), using countercurrent distribution technique, nisin probably contains at least two entities.

Tyrocidine.—The last general review of chemical work on tyrocidine appears to have been that of Synge (253) in 1948. Battersby & Craig (26, 27) have fractionated tyrocidine hydrochloride by extended countercurrent distribution and find that there is a family of three major components, A, B, and C. Tyrocidine A has been studied chiefly and purified to a stage where it seems to represent a cyclic peptide with the amino acid residue formula (D-phenylalanine)$_2$, L-phenylalanine, L-valine, L-tyrosine, L-leucine, L-proline, L-ornithine, L-glutamine, and L-asparagine, corresponding to the empirical formula $C_{66}H_{87}O_{13}N_{13}$. The empirical formula was well verified.

They suggest a general approach by means of the procedure given in detail including an amplified functional group analysis, all of which, it is thought may be useful in the characterization of higher molecular weight substances. Thus the molecular weight of tyrocidine A is of the order of 1300, or close to that derived from the empirical formula above, about 1270.

Antibiotics from Seed Plants

Dichroin.—A most interesting group of antimalarial antibiotics has been developed through studies which began with crude extracts of plants belonging to the genera *Dichroa* and *Hydrangea* of the family Saxifragaceae [Duggar *et al.* (82)]. After the active principle from *Hydrangea* was isolated in crystalline form by Ablondi *et al.* (1), it was determined as probably identical with that studied earlier from *Dichroa*. With a home-grown source of the alkaloid available, degradation work was carried out by Hutchings *et al.* (139), and extensive studies were made by Baker *et al.* (12 to 24), resulting in the synthesis of the alkaloid and of many related compounds. Throughout the study, assay of extracts and of all preparations were made against *Plasmodium lophurae* in ducks by Hewitt *et al.* (131).

The alkaloid is 3-[β-keto-γ-(3-hydroxy-2-piperidyl)propyl]-4-quinazolone with the structure indicated in formula VIII (p. 476). This alkaloid is identi-

cal with the active constituent of the Chinese plant known as Ch'ang Shan (*Dichroa febrifuga*). The quinine equivalent is approximately 100, and the *dl* form of the alkaloid is about one-half as active.

The results of assays of fifty-four of the synthetic quinazolones have been reported, including also the natually occurring *Hydrangea* alkaloid. Slight changes in structure have varied both effectiveness and therapeutic index of the compound. Substitutions at the five position of the quinazolone ring have afforded the better ratios with respect to activity and toxicity, a chlorine atom being particularly effective. Of the various substitutions at the 5, 6, 7, or 8 position, six had therapeutic indices at least twice that of the natural alkaloid, ten others being as effective as the latter, or nearly so. Of different batches of the natural alkaloid the suppressive dose varied between 0.1 and 0.2 mg./kg. The natural alkaloid for the investigations reported here was obtained from cultivated (usually greenhouse-grown or semi-hardy garden-grown) *Hydrangea* hybrids whose genetic make-up is unknown.

Steroid antibiotics.—Starting with dihydrotomatidin derived by hydrogenation from tomatidin, the aglycone of the alkaloid glucoside tomatin of *Lycopersicum pimpinellifolium*, Kuhn *et al.* (165, 166) have produced the diketo form which on hydrogenation and dehydration yields demissidin (IX, p. 476), which is the aglycone of the alkaloid glycoside in the leaves of *Solanum demissum*. This seems further to confirm the structure of tomatidin and also points to a close relationship of these antibiotics with solanin and solanidin of *Solanum sp.* According to Saito *et al.* (234) the degradation of tomatidin has on isolation yielded Δ^{16}-allopregnen-3(β)-ol-20-one, identified by comparison with authentic samples. This isolation is regarded as establishing the structure of the steroidal moiety of tomatidin (X, p. 476) as also attachment to the portion containing the secondary nitrogen.

The steroalkaloid and antibiotic solanocapsin has received renewed attention from Schlittler & Uehlinger (238) who have considered the empirical formula and the functional groups of the aglycone, the ring structure, also the structural isomerism between the base and its hydrochloride. They would modify slightly the empirical formula assigned by Barger *et al.*, so that it becomes $C_{27}H_{46}O_2N_2 \cdot H_2O$, thus associating this aglycone with solanidin, solasodin, tomatidin, etc. The isolation of 2-ethyl-5-methyl pyridine suggests a new structural formula which must be considered acceptable except for the location of the functional —NH_2 and —OH groups. See formula XI, p. 476.

Heartwood antibiotics.—Dating back several years, the earlier work of Erdtman, Gripenberg and others on steam-volatile products of the heartwood of certain gymnosperms has provoked continuing interest because of their chemical structure as well as of the antibiotic properties of many of the compounds. Gripenberg (102) reports a partial synthesis of the antibiotic thujic acid, achieved by the conversion of hexahydrothujic acid. Evidence is advanced in proof of the following formula for thujic acid, 4,4-dimethylcyclohepta-2,5,7-triene carboxylic acid (Formula XII, p. 476). From the order

Cupressales, Carlsson *et al.* (54) have isolated two steam volatile constituents from *Chamaecyparis nootkatensis* as follows: carvacrol, the sesquiterpene type "tropolone nootkatin," $C_{15}H_{20}O_2$, and chamic acid, a new substance, $C_{10}H_{14}O_2$. The presence of antibiotic tropolones in trees is especially interesting in view of the similar products from *Penicillia.*

Among other series of studies of extractives from the heartwood of trees are those of King *et al.* (159). From the West African tree *Distemonanthus benthamianus* was isolated ayanin, 3,7,4′-trimethylquercitin. Likewise, from another African tree known as muninga, *Pterocarpus angolensis,* King *et al.* (160) have obtained a product, termed muningin, the constitution of which is 6,4-dihydroxy-5,7-dimethoxyisoflavone.

Fulvoplumierin and others.—From *Plumeria acutifolia* (Apocynaceae) Grumbach *et al.* (111) have isolated a crystalline product given the name fulvoplumierin with the empirical formula $C_{14}H_{12}O_4$. This they found to be readily distinguished chemically from plumericin, which was obtained by Little & Johnstone (179) from *P. multiflora.* In fulvoplumierin the four oxygen atoms are described as distributed between a carbomethoxy group and an unsaturated lactone ring. Antibiosis *in vitro* is proved against selected gram-negative and gram-positive bacteria. At concentrations 1–5 μg/cc. the product inhibits the growth *in vitro* of various strains of *Mycobacterium tuberculosis.* There are obviously some properties suggesting a close relationship with plumierin.

The antibiotic (alkaloid) sanguinarin was found by Johnson *et al.* (149) to be fairly active against several species of *Escherichia* and *Aerobacter,* a decreasing order of toxicity being found for the two other plant products, berberin and physostigmin.

For future applications, it is encouraging to note that in the search for antibiotics several series of surveys are being continued, or are in early progress, looking toward a more systematic determination of the presence of antimicrobial substances in the seed plants. The references here noted are merely indicative of this activity [Azarowicz *et al.* (10); Hughes (138); Bishop & MacDonald (42); Winter & Willeke (285)].

Antibiotics in Animal Products

It would be impracticable to attempt a review of most of the current work dealing with antibiotic materials derived from intestinal worms, insects, crustaceans, and other invertebrates. Fortunately, the subject has been well handled currently by one who is an investigator in that field. Pavan (211, 212) has in the first-cited paper stressed particularly the products of intestinal worms, blood-sucking mites or insects, and certain other forms. In the other paper particular attention is given to the products of insects, including also his own research work. Active substances are considered from other classes of invertebrates, including myriapods and the scorpion. The literature cited is extensive.

A somewhat controversial problem is posed by studies on antibiotic substances which may be present in blood. Continuing earlier work [Anigstein *et al.* (6)], Micks *et al.* (191) prepared crude sanguinin and used it in experiments designed to protect mice against an injection of the beta-hemolytic strain of *Streptococcus zooepidemicus* given intraabdominally. The sanguinin treatment, by subcutaneous injection, began generally one to four days prior to infection, and the time-dosage relationship varied. Altogether the hydrolysate sanguinin was given *in vivo* to 268 mice, with 221 untreated controls for comparison. It is considered that a suppressive effect on the course of the infection was achieved in all the treated series. The more significant increase in survival occurred in the pretreated series. Sanguinin inhibits various bacteria *in vitro*.

The presence of a thermostable dialyzable substance with bacteriostatic and bactericidal activity towards tubercle bacilli, in human urine seems to have been established by Björnesjö (43) and a little later in certain animal tissues by Dubos (81) and independently by Björnesjö (44) in tissues of the cow. The conspicuous physical properties of the substance noted are: thermostability, resistance to acid and alkali, adsorption on activated charcoal, elution from charcoal with acetic acid, solubility in water and insolubility in most organic solvents. Bacteriostatic action *in vitro* is conditioned by somewhat higher concentration than occurs in normal urine.

Antibiotics in Animal Feed Supplements

Farm animals.—Research in the field of antibiotic feed supplements for young animals is not yet three years old, but the practice of using such supplements in the case of animals with simple stomachs, as in poultry and pigs, seems to be attaining rapidly the rank of a general procedure, even though in any given case the young animals display no recognizable symptoms of any infection and the surroundings offer no indications of unsanitary factors. As we interpret the findings, under acceptable conditions and with approved and tested antibiotics, this practice has resulted generally in enhancement of growth, less intestinal disturbances, often earlier marketing possibilities, and generally increased feed efficiencies, besides posing new and important problems in the chemistry of nutrition. Available references of the current year are such as those of Briggs (48), Cuthbertson (72), and an editorial article in Nutrition Reviews (288).

Current investigations in the United States, fostered in part by the agricultural experiment stations (see work in progress at Cornell University, Kansas State College, Louisiana State University, and Iowa State College, among others) are pressing forward for further data on antibiotic feeding in all classes of ruminating animals. Rusoff *et al.* (233) and Knodt *et al.* (162) reported on preliminary work as indicating reasonable benefits from the use of antibiotic supplements with dairy calves, and MacKay *et al.* (182) report improved growth with similar animals, even though in these tests scours was

was not present as a factor that might add greater importance to the antibiotic. Lower levels of the antibiotic than were formerly used have been found satisfactory by Jordan & Bell (153) and Jordan (152) in the diets of suckling lambs and feeder sheep. Neumann *et al.* (206) have attempted to assess the possible value of feeding small amounts of supplements in the ration for fattening beef cattle, but definite recommendations are not available. The antibiotics chiefly concerned thus far in such tests are aureomycin, penicillin, streptomycin, terramycin, and bacitracin. A new device in furnishing baby pigs antibiotics, during the time they eat no appreciable amounts of feed, is that of implanting pellets subcutaneously at the base of the ear. Apparently, the first trials were made with bacitracin only [Pensack & Gerard (213)].

Controversial reports have continued in respect to the mode of action of antibiotic supplements. Apparently strong is the evidence supporting the view that the effects are commonly achieved through action on the intestinal flora either by suppression of infection-inducing bacteria or by effecting favorable changes in the relative numbers of the components of the flora. On the other hand, a few reports indicate little or no action on the intestinal flora, referring rather to vitamin sparing, enhanced protein utilization [Anderson *et al.* (3)], or increased nutrient absorption. Recently, however, a boost for the significance of the intestinal flora has been given by statements which have become public as a result of the colloquium held June 4, 1952, at the Lobund Institute, University of Notre Dame and available to us without the basic data. These statements indicate that averaging the results there were no positive (growth enhancing) effects when germ-free chicks were fed terramycin or procaine penicillin. In the case of streptomycin, chloromycetin, and bacitracin there were slightly diminished growth rates. Germ-free turkey poults did not respond to procaine penicillin.

Some experiments with the rat.—With the rat as test animal in the experimental work, Sauberlich (236) obtained results indicating that with the addition of 0.01 per cent penicillin there was marked stimulation of growth only when the diet was free from, or low in, thiamine, pyridoxine, pantothenic acid, and to some extent riboflavin. The response to aureomycin was similar when there were thiamine and pantothenic acid deficiencies. However, in basal diets completely vitamin supplemented the antibiotics had no measurable growth influence in the rat. Continuing earlier studies on effects of large doses of cortisone, Meites (190) used 50 young albino rats in five groups, all being maintained on a casein-glucose diet and all except a control lot given each day for 28 days injections of cortisone acetate (1 mg. daily). Three lots received respectively vitamin B_{12}, aureomycin, and a combination of these. Cortisone alone induced growth inhibition, alopecia, and thymus gland atrophy; cobalamin (vitamin B_{12}) at 200 μg/kg of diet or aureomycin (0.005 per cent) largely overcame these effects, combination of the two being more effective. This substantiates earlier work and suggestions of Meites (189).

Bacteriological Studies in Relation to Sensitivity, Resistance, Synergism, and Mode of Action

Sensitivity.—Sensitivity determinations have been most valuable procedures in the comparative study of the antibacterial properties of antibiotics. These determinations are commonly *in vitro;* also they are often made by the agar plate method, and the latter thus becomes the basis of the usual "antibiotic spectrum." Changes in media or in any component of the environment, or differences in diffusibility of the antibiotics, may greatly affect comparative values of sensitivity or resistance. Numerous studies that may not be discussed here, for lack of space, deal with these factors. For the more exacting *in vitro* specificity determinations, that is, such as may be more reliable and helpful in selecting an antibiotic for a given treatment, the serial dilution tube test is now apparently a requisite. However, no type of *in vitro* determination can give the final answer for therapeutic practice if it is practicable to arrange *in vivo* susceptibility tests.

A timely report by Jackson & Finland (144) is of special interest in respect to sensitivity values not merely because of the diversity of factors well considered but also because of emphasis on the special circumstances of the clinical applications. Seven bacteria and six antibiotics are tested by several methods. It is found that values are profoundly influenced by the test conditions, and one further important consideration is emphasis on a determination of the "true bacterial incitant" of any given infection. When this is established, it is indicated that for most infections therapy may be then selected intelligently. Sensitivity tests are more often needed where one is concerned with causative organisms, different strains of which display differential behavior respecting susceptibility to the antibiotics under consideration.

In spite of the difficulties necessarily involved, Schneierson (240) has attempted to follow changes in bacterial sensitivity to aureomycin and chloramphenicol occurring in the hospital during the past three years [see also Koch & Bourgeois (163)]. Using aureomycin, nisin, penicillin, and streptomycin, Edwards (86) has investigated both *in vitro* (bacteriostatic and bactericidal) and *in vivo* action. For the *in vivo* action, protection against four different experimental infections in mice constituted the test. Between *in vitro* sensitivity and *in vivo* susceptibility, there was no strict correlation. Accordingly he states that *in vivo* tests should confirm selection of antibiotics for therapeutic use clinically. Compare also the work of Bliss & Todd (45), Villemin *et al.* (266), and Lund (180).

Resistance.—The problem of the resistance of bacteria to antibiotics, and more particularly acquired resistance, received careful consideration and therefore a liberal space allowance in the last year's review [Work (286)]. Similarly, the discussion of the mechanism of action of antibiotics received attention commensurate with its chemical significance and interest. There has been no cessation of research effort nor of result in either of these areas,

but it may be assumed that the field has actually grown in complexity because of the increased number of antibiotics as well as by reason of the diversity of structures involved. In any case, the treatment of these phases for the present year is closely limited. Many observations support the idea of a trend toward increased incidence of cultures resistant to the clinically important antibiotics. Although dire consequences of this trend have been forecast and some danger probably does exist, it seems that resistant variants of normally sensitive organisms are not yet an important clinical problem except (*a*) in the case of organisms producing penicillinase or (*b*) with streptomycin and related antibiotics to which a high degree of resistance may occur very rapidly.

In many cases the resistant form of an organism grows more slowly than the normal form, thus acquired resistance may tend to be lost in a natural population. Cross resistance does occur and may be complete, partial, or one-way. As an example, results obtained by Szybalski & Bryson (255) may be cited. Production of resistance in strains of *E. coli* to 15 antibiotics was studied. Viomycin, vinactin, neomycin, catenulin, and streptothricin produced reciprocal cross resistance and also resistance to streptomycin, but streptomycin-resistant cultures were not necessarily much more resistant to all members of this group of antibiotics. Aureomycin and terramycin frequently exhibit reciprocal cross resistance, likewise chloramphenicol, depending, however, on the organisms involved. The groups of antibiotics which do not produce cross resistance to each other probably act on microorganisms via different essential reactions. A combination of antibiotics with different modes of action may bring out a synergistic effect, and one possible explanation is to regard the mechanism as a combined attack on alternative metabolic pathways.

Synergism.—In papers which present both the side of current theories and some new experimental evidence Miles *et al.* (192) and Jawetz *et al.* (146) support the view that commonly synergism requires the simultaneous action of both members of a synergistic pair. The pairs selected are known to display synergism, under certain circumstances; these are penicillin and streptomycin against *Streptococcus faecalis*, and terramycin and bacitracin against *Staphylococcus albus*, all *in vitro* tests. Diagrams are used to demonstrate results in the two systems and to clarify discussion. The results are interpreted to indicate that antibiotic synergism under these conditions is observed only when both members of the pair are acting simultaneously on the population and not in sequence. These findings tend to favor the view that synergism involves simultaneous blocking of alternate enzyme pathways; at the same time, we think, it does not preclude the possibility that some other mechanisms may also participate in the operation. A suggestive scheme of combined antibiotic action has now been elaborated [Jawetz & Gunnison (145)].

Mode of action.—Little new definitive information has been published on the mode of action of antibiotics since last year [see Work (286)]. Also the subject has been reviewed in some detail by Kirby (161) and by Pratt &

Dufrenoy (220). Osteux & Laturaze (207) found that aureomycin, terramycin, or chloramphenicol inhibited the pyruvic-hydrogen-lyase system of *Clostridium welchii*. This inhibition was hindered by the presence of biotin. Osteux *et al.* (208) found, however, that these antibiotics did not seem to act as antibiotin in the anaerobic deamination of serine, but rather inhibited the operation of the citric acid cycle at about 0.0005 *M* as compared with malate at 0.03 *M* minimum concentrations. Interesting experiments by Swendseid *et al.* (252) have shown that leucocytes produce a "hemofolin" when incubated with folic acid. This material can reverse chloramphenicol inhibition, and a possible mode of action of chloramphenicol may lie in the inhibition of some step in folic acid metabolism. Potter & Reif (218) have presented in greater detail the studies on the inhibition of the succinoxidase system by antimycin A referred to last year [Work (286)]. Muira *et al.* (200, 201) have found that the mode of action of usnic acid is related to oxidative phosphorylation and studied the effect of terramycin on phosphate metabolism of *Staphylococcus aureus*.

Antibiotics in Phytopathology

With the unparalleled developments in respect to the use of antibiotics in human medicine and animal economy it was to be expected that the possibilities of using such antibiotic agents in plant protection would soon be thoroughly explored. Activity on the part of phytopathologists in this field exceeds any expectations. It represents, however, a renewal of effort, since the discovery of gliotoxin by Weindling (275, 276) in 1932 was one other occasion when interest was aroused. Nevertheless, the successful or practicable use of such chemical agents in plant production has limitations broader than those of acute and chronic toxicity, so important in the human and animal side. Among the intimated limitations on the plant side, some major ones are stability and cost of the antibiotics; many minor restrictions are related to the mode of application of the antibiotic, as in soil treatments, vegetational treatments, or seed treatments; while still other considerations apply in different situations such as greenhouse cultures, intensive gardening, and extensive field operations.

In testing the activities of nine antibiotics, including none of those therapeutically prominent, Wallen & Skolko (270) used as a criterion spore germination in *Ascochyta pisi*, a disease incitant of the garden pea. It was established that five of the antibiotics were fungistatic at 20 p.p.m. or less, and all were fungicidal at 100 p.p.m. or less. Only one (antibiotic XG) gave adequate control in seed treatment. Wright (287) used thirteen antibiotics in comparison with 3-indolylacetic acid and coumarin, tested against percentage seed germination of wheat. Referring now for a moment to merely one of the concentrations used, 5 p.p.m., the results show relatively no toxicity, or seed damage. However, with seeds of white mustard there was some injury, but more from the indolylacetic acid than from the more toxic antibiotic, gliotoxin. In fact, at higher concentrations indolylacetic acid was generally

more toxic than the antibiotics, coumarin less so. Against the bacterial-induced disease "fire blight of apple," and working with rather small-scale operations, Murneek (204) finds thiolutin fairly effective. The essential results were: control, 224 infections per tree; streptomycin, 94; and thiolutin 48 infections. The concentrations of these last two were respectively 10 and 19 gm. per gallon. Gottlieb & Siminoff (99) have indicated that in certain stability determinations with chloramphenicol the antibiotic might be added to the soil at the rate of 50 μg/gm., and yet it would be rapidly degraded by the soil population. Martin & Gottlieb (185) later, working on soil relations of aureomycin and terramycin, found, as have several others, that moderate concentrations of these antibiotics are completely removed from aqueous solution by the soil. The antibiotic, helixin, as a partly purified preparation was tested in certain greenhouse experiments by Leben & Keitt (171) on tomato foliage, as a spray application against *Alternaria solani*. LD_{50} and LD_{95} values were respectively 6.5 and 37.9 μg/cc. The antibiotic was an effective protectant, and there was no foliage damage when used as a spray.

With seeds of a variety of spring wheat naturally as well as artificially inoculated (1 part spores to 200 parts seed), Henry *et al.* (129) tested the effect of actidione on (*a*) seedling emergence and on (*b*) percentage of smutted heads on the greenhouse-grown plants. Actidione was used as dust ($\frac{1}{2}$ oz./bu.) and as aerosol-liquid (10 p.p.m.) with adequate controls. Substantial prevention was achieved, the diluents showing influence in this effect. Primary leaves of bean plants inoculated with the halo blight organism *Pseudomonas medicaginis* var. phaseolicola failed to develop disease symptoms, according to Mitchell *et al.* (193), when a small amount of streptomycin sulfate was spread on the young stems previous to inoculation. Moreover, the antibiotic was translocated to trifoliate leaves. Aureomycin and terramycin reduced the severity of symptoms only. However, the acidity of the pastes applied may be a consideration. Eight other antibiotics failed to affect disease development. The above data and indications are merely suggestive of the status of antibiotics in plant pathology.

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THE BIOCHEMISTRY OF VISION[1,2]

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It is ten years since this subject was last reviewed in these pages by Hecht (44).[3] Some of those years were wasted by the war. Those who think that war stimulates scientific research are not scientists. It may stimulate technology. All the organized projects, the money and material poured into them, the committees and conferences, succeed mainly in obscuring the fact that little of scientific importance is being accomplished. One of our problems is to keep these conditions from continuing throughout the peace.

The end of the war marked a turning point in this field, for since that time the chemistry of vision has been brought to a new level. Ten years ago it was already clear that three light-sensitive pigments are found in the retinal receptors: rhodopsin and porphyropsin in rods, iodopsin in cones. All three pigments had been recognized to be carotenoid-proteins, proteins bearing carotenoid prosthetic groups to which they owe their color and sensitivity to light. On exposure to light, the pigments bleach, and the carotenoid is freed from the protein. In the rhodopsin system these changes had been shown to take the form: rhodopsin $\overset{\text{light}}{\rightleftarrows}$ retinene+protein→vitamin A +protein→rhodopsin (90, 91, 92).[4] In the porphyropsin system, they have a precisely analogous form, but involve different carotenoids: porphyropsin $\overset{\text{light}}{\rightleftarrows}$ retinene$_2$+protein→vitamin A$_2$+protein→porphyropsin (94, 98). The structure of the iodopsin system was not yet known, though there was indirect evidence that it might well include vitamin A (95, 101).

This skeleton of reactions has since been clothed with chemistry. The structures of the visual carotenoids are now known, the enzymes and coenzymes are identified, and virtually all the reactions in all three systems have been carried out in solution.

I think this brings the field to another turning point. We still know very little concerning the visual proteins, what we now call the opsins, the nature of the linkages between them and the carotenoids, and the protein properties of the visual pigments themselves. I think that the major effort of the

[1] The survey of the literature pertaining to this review was concluded in January, 1953.

[2] The following abbreviations are used: DPN for diphosphopyridine nucleotide; DPNH$_2$ for diphosphopyridine nucleotide (reduced form).

[3] Various aspects of this subject have recently been reviewed by Broda (16), Granit (37a, 39), Morton (68, 69), von Studnitz (89), Tansley (85), and Wald (102, 106, 109, 110).

[4] Throughout this review the terms vitamin A and retinene refer to the substances otherwise called vitamin A$_1$ and retinene$_1$.

next few years will lie as much in the protein as in the carotenoid chemistry of vision.

It should be remembered also that however stimulating one may find the exploration of visual substances and reactions, the ultimate problem toward which all of this must turn is how these processes lead to a nervous excitation, how they initiate the train of neural events that ends in seeing. This is the real task that lies ahead, and one can hope nothing better for this field than that the coming years will make progress with it.

Rhodopsin and Porphyropsin

Rhodopsin is the bright red, light-sensitive pigment discovered in the rods of the frog retina by Franz Boll in 1876. Porphyropsin is a similar pigment, purple in color, found characteristically in the rods of freshwater vertebrates: freshwater fishes, certain amphibia, and cyclostomes (102). Its history extends back to Kühne, but it was recognized as a distinct pigment and named only recently (94, 98). Exposed to light in the retina, both these pigments bleach over orange intermediates to yellow, and finally to colorless products.

Concerning names.—A comment on the naming of the visual pigments may not be amiss. Franz Boll originally called the pigment of the frog retina *Sehrot*, visual red. In the following year Kühne renamed it *Sehpurpur*, seeing in it a faint nuance in the direction of purple, and visual purple it became. This made matters difficult later, when one had to find a name for the photosensitive pigment of the freshwater fish retina, which is unmistakably purple. I suggested therefore that we adopt Kühne's other name for the frog pigment, *rhodopsin* (Greek *rôdon* = rose) and call the purple pigment of freshwater fishes porphyropsin (Greek *pôrphyros* = purple). When still later I found the cone pigment of the chicken to have an absorption spectrum that inclines its color toward violet, I suggested that it be called iodopsin (Greek *ion* = violet).

Hecht preferred to translate iodopsin into the homelier term, visual violet. Recently, however, Granit (37a) has begun to call porphyropsin visual violet, though one has only to look at this pigment to see that it is not violet. This practice has since been taken up by several English workers. In consequence they call rhodopsin, which to most persons looks red, visual purple; and porphyropsin, which is distinctly purple, visual violet. What then to do with iodopsin? This is called visual blue (cf. 32)!

One of the greatest services that Lavoisier performed for chemistry was to give it a technical vocabulary. Blue vitriol became copper sulfate; gray calx of antimony, antimony oxide; spirit of wine, alcohol. With this one could pass from alchemy to chemistry. I venture to think that the time has come to make that transition in the field of vision.

This has been in part the nature of my objection to even such descriptively accurate terms as Kühne's visual yellow and visual white (58) and

Lythgoe's transient orange and indicator yellow (64). As descriptions of states of a retina or an extract, such terms may be useful for a time; as designations of substances in an expanding field of biochemistry, they are inadequate. They evade the basic question, whether they refer to substances or mixtures, and they do not lend themselves to the further development of the subject. For example, from rhodopsin one can go on to designate the related substances, lumi-, meta-, and iso-rhodopsin. All these substances have nearly the same color. What would one have done with "visual purple"?

Preparation.—Soon after the discovery of rhodopsin, Kühne (58, 59) found that it can be extracted into aqueous solution with the aid of bile salts, even after most other retinal proteins had been rendered insoluble by treatment with alum. Until a few years ago, the methods in use for preparing solutions of rhodopsin were still those of Kühne, but much effort has gone recently into improving them.

It should be understood that the problem of obtaining pure rhodopsin is complicated in two ways: the necessity to use a solubilizer such as bile salts and the extremely small quantities in which the pigment can be obtained, which preclude any possibility of using conventional protein procedures to purify it. Once rhodopsin is in solution, little can be done for it. The point is to remove as many potential impurities as possible before extracting it from the retina.

A long step in this direction, and a valuable procedure for many types of study, involves the separation of the outer segments of the rods from the remainder of the retina [Lythgoe (64)]. This is achieved by shaking or macerating the retina and separating out the rods by filtration or differential centrifugation. Saito (79) has shown that the rod outer segments can be floated out by centrifuging in about 45 per cent sucrose, the remaining retinal tissues sedimenting to the bottom of the vessel. In variations of these procedures, one can obtain dense suspensions of rod outer segments, virtually alone (117). In the frog, where they are unusually large, they measure about 6×50 micra. Within this small space they enclose the entire photoreceptor process, about as high a degree of isolation of a physiological function as the organism affords.

The procedures now in use for preparing rhodopsin include the separation of the rod outer segments; their hardening in 4 per cent alum to render other proteins insoluble; extraction with petroleum ether to remove fat-soluble substances; leeching with aqueous buffer solutions to remove water-soluble substances; and finally extraction of rhodopsin from the residue with the aid of such a solubilizing agent as crystalline digitonin (27, 77, 113). Except for the alum treatment, the procedure is very similar to that introduced recently for extracting cytochrome oxidase and other heme pigments from cell particles (cf. 84).

Exactly the same procedure is used to prepare porphyropsin, or opsin, the protein moiety of both visual pigments (112, 113). To prepare rhodopsin

or porphyropsin, one begins with dark adapted retinas and carries out all operations in dim red light. To prepare the opsins, one uses light adapted or wholly bleached retinas and works in ordinary light.

Absorption spectra.—The absorption spectrum of rhodopsin in aqueous solution consists of three bands: the α-band, maximal at about 500 mμ, upon which rod vision principally depends; a very small β-band in the near ultraviolet, at about 350 mμ; and a tall, narrow γ-band at about 278 mμ. The α- and β-bands go with the carotenoid prosthetic group, the γ-band with opsin. The latter is the typical protein band found in all proteins which have aromatic amino acids in their composition. Preparations of purified opsin exhibit the protein band alone (55). Light absorbed in the α- and β-bands bleaches rhodopsin and can be seen. Light absorbed in the protein band, however, does not appear to be available for bleaching; as the absorption spectrum of rhodopsin rises to the high γ-peak, its sensitivity to light falls to a low level (37, 106, 110).

Porphyropsin exhibits a similar trio of absorption bands. The maximum of the α-band lies at about 522 mμ, the β-band at about 378 mμ, while the protein band is in its usual position (94, 109, 110).[5]

Rhodopsin, like other proteins, varies somewhat from one animal species to another. These differences are reflected in small displacements in its absorption spectrum. So, for example, the α-band maximum lies at about 498 mμ in cattle, rats, and dogfish; 500 mμ in sheep; and 502 mμ in certain frogs (27, 96, 109). In the first rhodopsin to be isolated from an invertebrate, the squid, the displacement is greater: the α-band lies at 490 mμ, the β-band at about 365 mμ, and the opsin band is in its usual position (78, 109). Like the hemoglobins, each of these pigments contains the same prosthetic group; it is the protein or the attachment to protein that varies. The term rhodopsin, or opsin, therefore, like hemoglobin, designates a family of closely related substances, each of which should be named for the animal of origin: cattle rhodopsin, squid rhodopsin, frog opsin, and so on.

Purity.—In evaluating rhodopsin preparations, the ratio of the extinctions at 400 and 500 mμ (400/500 ratio) provides a useful index of what may be called "optical purity" (96). The best preparations of frog and cattle rhodopsin possess 400/500 ratios of 0.22 to 0.26 (27, 106, 109). Goodeve, Lythgoe & Schneider (37) found rhodopsin in solution to have sensitivities to light at 400 and 500 mμ corresponding to a 400/500 ratio of 0.22. It has been estimated also that the 400/500 ratio of pure rhodopsin can not be less than 0.20 (96). It must be concluded that the best solutions of rhodopsin

[5] Dartnall's argument (33) that the α-band maximum of porphyropsin is at 533 ± 2 mμ rather than at 522 ± 2 mμ (98) is without foundation. His measurements on the tench retina, which show an ill-defined maximum at about 495 mμ, must be weighed against those of Kampa (56), which find it at 520 mμ; and his report that in the pike it lies at about 530 mμ must be weighed against that of Collins & Morton (27), who find the maximum at about 525 mμ. All the measurements from our laboratory find porphyropsin in the neighborhood of 522 mμ.

which have been prepared have absorption spectra in the visible region very close to that of the pure pigment.

A similar ratio of the absorptions at 278 and 500 mμ (278/500 ratio) provides a criterion of optical purity in the ultraviolet and indicates also the proportion of protein to chromophore in various rhodopsin preparations. The lowest such ratio so far reported has been 2.2, found equally in cattle and bullfrog preparations (109). There is as yet no assurance that this approximates the ratio for pure rhodopsin.

Bleaching.—On exposing rhodopsin in solution to light, it bleaches, first to unstable orange material, then to a final yellow mixture of retinene and opsin, for the most part still loosely bound to each other (96). Only the first process is photochemical; the second is an ordinary thermal, i.e., "dark" —reaction (64, 93). Lythgoe called the initial orange product "transient orange," the final product "indicator yellow." I have stated above the reasons for my reluctance to use these terms, which in any case are known now to refer to mixtures rather than single substances.

Broda & Goodeve (17) were the first to isolate the light reaction in the bleaching of rhodopsin, by exposing this pigment to light at the temperature of dry ice (-70°C. to -80°C.), at which "dark" reactions are almost at a standstill. Their most interesting observation was that under these conditions the irradiation of rhodopsin merely causes its spectrum to shift about 5 mμ toward the violet, with little change in height. The remainder of bleaching is caused by thermal reactions.

Such experiments have recently been performed with more adequate spectrophotometric procedures (29, 115). On exhaustive irradiation at -40° to -100°C., the α-band of rhodopsin shifts about 5 mμ toward shorter wavelengths and rises (cattle) or falls (frog) slightly in height. The color changes very little, from red to orange-red, with little change in depth. This is as far as light alone brings the bleaching of rhodopsin. Its product is called *lumi-rhodopsin* (115).

On warming in the dark to temperatures in the neighborhood of -15°C., lumi-rhodopsin undergoes a small further shift of absorption spectrum toward the violet, still with little change in color, yielding *meta-rhodopsin.* On warming to room temperature in the dark, meta-rhodopsin goes over to a mixture of approximately equal amounts of retinene+opsin and regenerated rhodopsin.

Many years ago Kühne (58) observed that dry rhodopsin does not appear to bleach, even in direct sunlight. It has now been shown, however, that in the dry state rhodopsin does undergo the light reaction, though with so little change in color as to have escaped notice. Rhodopsin prepared in dry gelatine films possesses an absorption spectrum much like that in solution. On exposure to light it yields lumi-rhodopsin, and at room temperature in the dark this goes over to meta-rhodopsin. Meta-rhodopsin is stable indefinitely, if kept dry, but on wetting in the dark, it bleaches to a mixture of approximately equal parts retinine+opsin and regenerated rhodopsin (115).

The rhodopsin regenerated under these circumstances has its absorption spectrum displaced slightly toward shorter wavelengths than that of the original pigment. Collins & Morton (29) suggested for this reason that it be called "isorhodopsin." It has proved to be a mixture of natural and "slightly altered" rhodopsin (109); the nature of the "alteration" is discussed in the section on *cis-trans* isomers below.

Squid rhodopsin bleaches much as does vertebrate rhodopsin, though here the dark changes are much slower. Bliss (11) has described a pigment obtained from the squid retina which he called "cephalopsin," which was unaffected by light though it decomposed to retinene in the dark. Bliss, however, had made his preparations in daylight. What he described was in fact meta-rhodopsin. Squid rhodopsin, like that of vertebrates, is relatively stable in the dark. On exposure to light it yields lumi-rhodopsin, which is converted in light or dark to meta-rhodopsin. In the dark, at temperatures above 20°C., meta-rhodopsin goes over to a mixture of retinene+opsin and regenerated rhodopsin (78).

These experiments reveal a striking correspondence between the bleaching of rhodopsin and the photographic process. In both cases light forms a "latent image," involving little visible change. Large changes in color are the result of dark processes, i.e., of "development." The parallel is particularly close in the case of dry gelatine films of rhodopsin, in which exposure to light yields a stable "latent image" composed of meta-rhodopsin, which can be developed at any later time simply by wetting (108, 115).

Energetics.—It has been reported that ultraviolet light (254 mμ), absorbed in the protein moiety of rhodopsin, is relatively ineffective in bleaching the molecule (37). The implication is that in rhodopsin, energy can not be transferred efficiently from the protein to the prosthetic group. This is very different behavior from that reported for myoglobin, in which ultraviolet light absorbed in the protein appears to be very efficient in splitting carbon monoxide from the prosthetic group (19). The ultraviolet sensitivity of rhodopsin should be re-examined, for other studies imply that in this molecule also energy is transmitted freely from the protein to the chromophore (77).

Hecht (42) showed some years ago that in white light rhodopsin bleaches at a rate which is independent of the temperature; that is, its activation energy is supplied by the quanta of absorbed light alone. On the other hand, rhodopsin can be bleached also by heating in the dark, with an apparent energy of activation of about 44 kcal. per mole, supplied entirely by the internal energy of the molecules (66). Forty-four kcal. corresponds to the energy content of a mole of quanta (i.e., 6×10^{23} quanta) of wavelength 650 mμ. St. George has now found experimentally that the bleaching of rhodopsin, which is independent of the temperature over most of the visible spectrum, acquires a temperature coefficient at about 600 mμ, which increases with further increase in the wavelength. That is, above 600 mμ, the quanta of absorbed light become too small to supply the entire energy of activation,

and larger and larger supplements of internal energy are needed to activate the molecule. The sum of the energy of the absorbed quantum and the internal energy remains constant at about 48.5 Kcal. per mole (77). Rod vision has been shown to extend to 1050 mμ (116); at this wavelength, at which the energy content of a mole of quanta is only 27 Kcal., it would seem that about 44 per cent of the activation energy for bleaching rhodopsin is supplied as heat.

Rhodopsin as protein.—It may seem strange to persons outside this field that we still have no assurance of having prepared pure rhodopsin, and still do not know some of its most elementary molecular properties. Reasons for this have already been noted: the small quantities of rhodopsin which can be obtained at best; but more important still, the fact that this molecule, as also its protein moiety opsin, can be brought into solution only with the aid of a solubilizer. What exists in solution therefore is never free rhodopsin or opsin, but complexes of these molecules with the solubilizer.

Such a solubilizer as digitonin, even when alone, forms large micelles in solution. Smith & Pickels (82) found that digitonin sediments in the ultracentrifuge with a sharp boundary, as though monodisperse, with an apparent particle weight of at least 75,000, indicating at least 60 molecules of digitonin per micelle. It is difficult therefore to evaluate the information that frog rhodopsin in digitonin or bile salts solution sediments in the ultracentrifuge with apparent particle weights of about 270,000 (48) or about 100,000 (65). What portion of these particle weights is composed of solubilizer is wholly problematical. Diffusion measurements on rhodopsin, which have yielded an estimate of particle weight of about 810,000 (45) are subject to the same error.

It must be conceded that as yet we have no idea of the molecular weight or dimensions of rhodopsin. By the same token we have no idea how many chromophore groups each rhodopsin molecule possesses. Even the measurement of isoelectric point, which has yielded values of 4.5 (98) and 4.47 (18) for rhodopsin, and 4.57 for its bleached product (18), may be seriously disturbed by this factor, for micelles of digitonin or bile salts are themselves strongly charged. I think there is little doubt that with patient effort some of these difficulties can be circumvented, but no serious effort has yet been made to do so.

Bleaching and denaturation.—One of the principal reasons offered originally for the belief that rhodopsin is a protein was that a great variety of protein denaturation procedures all bleach this molecule (91). Shortly afterward Mirsky (67) suggested that even under normal circumstances the bleaching and regeneration of rhodopsin can be regarded as a reversible denaturation. This seemed to me at the time a gratuitous view (96); yet the more we have learned since of the process of bleaching, the more it has come to resemble a denaturation.

So, for example, the bleaching of rhodopsin by heat, in some ways a typical heat denaturation, has been shown to merge its properties imperceptibly

with bleaching by light (77). Bleaching is reported to shift the isoelectric point of rhodopsin from 4.47 to 4.57 (18); such small displacements of isoelectric point in the alkaline direction commonly accompany denaturation. Rhodopsin, though it goes to meta-rhodopsin in the dry state, requires water to bleach (115); the need for water in protein denaturation was demonstrated by Chick & Martin (25). The bleaching of rhodopsin has recently been shown to expose new sulfhydryl groups, an effect characteristic of many protein denaturations (113).

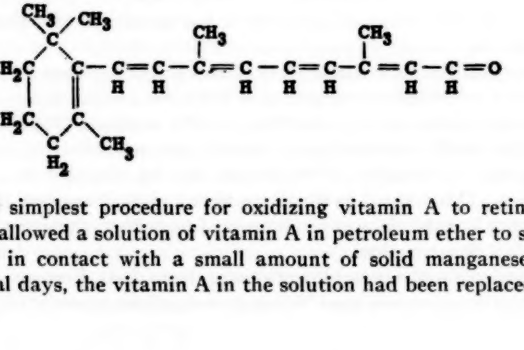
The regeneration of rhodopsin from retinene and opsin is a spontaneous, i.e., an energy-yielding reaction. It is the bleaching of rhodopsin that requires energy (112). Similar relations have recently been demonstrated in a variety of proteins. Over a range of temperatures, an equilibrium exists between native and denatured protein, which below a specific temperature favors the native state. Below this critical temperature, denatured protein goes over spontaneously to the native condition, and conversely work must be done to denature the protein.

It can hardly be doubted that these are meaningful relationships; and one may hope to draw from them eventually some measure of clarification, both of the behavior of rhodopsin and of the nature of the denaturation process.

Retinene

One of the strongest stimuli to progress in this field in the post-war period came from the clarification by Morton and his colleagues at Liverpool of the structure of retinene, their isolation of this substance in crystalline condition, and their discovery of simple procedures for preparing it from vitamin A (3, 4). Up to this point, retinene could be obtained only in microgram quantites from dark adapted retinas, dissected from the eye in dim red light. Now it can be prepared from vitamin A in any quantity desired, with the simplest of means, and by a procedure that occupies only several minutes.

Vitamin A is the primary alcohol, $C_{19}H_{27}CH_2OH$. Retinene is the corresponding aldehyde, $C_{19}H_{27}HC{=}O$:



In their simplest procedure for oxidizing vitamin A to retinene, Ball *et al.* (3, 4) allowed a solution of vitamin A in petroleum ether to stand in a refrigerator in contact with a small amount of solid manganese dioxide. After several days, the vitamin A in the solution had been replaced by retinene.

On re-examining this process, Wald (104) found it to take the following course. Manganese dioxide adsorbs vitamin A very strongly from solution, and in the adsorbed state oxidizes it to retinene. Retinene is much less strongly adsorbed, however, and so is displaced from the manganese dioxide surface by new vitamin A, as fast as it is formed. In this way all the vitamin A passes over the manganese dioxide and is replaced in the solution by retinene.

The final charge of vitamin A on the adsorbent is oxidized to retinene, and then, with no further vitamin A present to displace it, is oxidized further to what appears to be a hydroxyretinene. This has been called the 545 mμ-chromogen, from the λ_{max} of the product which it yields in the antimony chloride test (104). It is important for this reason that to make retinene, a large excess of vitamin A be employed. If the relative amount of manganese dioxide is too large, it adsorbs all the vitamin A at once from solution and oxidizes it entirely to the 545 mμ-chromogen.

Once the nature of this reaction was appreciated, it was recast in the form of an ordinary chromatographic adsorption. A small amount of manganese dioxide powder is packed into a glass tube, and a solution of vitamin A in petroleum ether is drawn through this under light suction. A solution of retinene runs off as the filtrate (104). This can be purified further by adsorption on calcium carbonate or "weakened" alumina (4, 107).

The process described above has been referred to as a "chromatographic oxidation." The fact that a solid surface serves at once as adsorbent and reagent lends it a specificity and orientation otherwise to be looked for only in enzyme reactions. As with adsorbents in general (and enzymes) the condition of the reagent is of prime importance. Various samples of solid manganese dioxide vary greatly in activity; the amounts of manganese dioxide optimal for oxidizing a given amount of vitamin A may vary as much as 25 times (4).

One of the most significant differences between retinene and vitamin A is its enormously greater reactivity. Vitamin A is relatively inert; but retinene is available potentially for the whole gamut of addition reactions, condensations, oxidations, and reductions that go with the aldehyde group, particularly activated in this molecule by conjugation with a long, unsaturated hydrocarbon chain. Some of these reactions, apart from their intrinsic interest, have large significance for the role of retinene in vision.

Like other aldehydes, retinene condenses readily with amino groups in a wide variety of molecules—simple amines, proteins, and amino acids—to give Schiff bases of the following general structure (1): $C_{19}H_{27}HC{=}O$ (retinene) $+ RNH_2$ (amine) $= C_{19}H_{27}HC{=}NR$ (Schiff base) $+ H_2O$. An interesting characteristic of such complexes is that they change in color with pH, being almost colorless in alkaline solution (λ_{max} 360–370 mμ), yellow at neutrality or in very dilute acid (λ_{max} 380–390 mμ), while in stronger acid (0.1 *N* HCl) they form bright orange products with λ_{max} 435–460 mμ (1).

It has long been recognized that the product of bleaching rhodopsin

behaves as a pH indicator, almost colorless in alkaline and bright orange in acid solution. It is this property that attached to it the term "indicator yellow." Pure retinene does not change in spectrum with pH. The indicator property however is characteristic of the aliphatic amino complexes of retinene described above. There is little doubt that "indicator yellow" is this type of complex, formed by the condensation of retinene with the amino groups of opsin and other retinal molecules (2, 28).

Indeed retinene, on being added to aqueous extracts of the retina and other tissues, quickly forms such complexes. Their formation is made apparent by (*a*) the indicator behavior already described; (*b*) aqueous solutions containing amino groups with which retinene can condense, take up retinene without the aid of a solubilizer. In tissue extracts this function is performed primarily by proteins, and the retinene brought into solution in this way can be precipitated again with the protein fraction. (*c*) Retinene coupled in this manner is extracted with fat solvents only with great difficulty when the solutions are alkaline, a condition which favors coupling with amino groups, and is extracted more and more easily the higher the acidity (107).

One of the most useful complexes of this nature is retinene oxime, formed by the condensation of retinene with hydroxylamine. This reaction occurs almost instantaenously at room temperature in water solution and has been of great utility in the analysis of visual processes (112, 118): $C_{19}H_{27}HC{=}O$ (retinene) $+NH_2OH$ (hydroxylamine) $=C_{19}H_{27}HC{=}NOH$ (retinene oxime) $+H_2O$. Unlike other amino complexes of retinene, the oxime does not change in spectrum between pH 4 and 9.

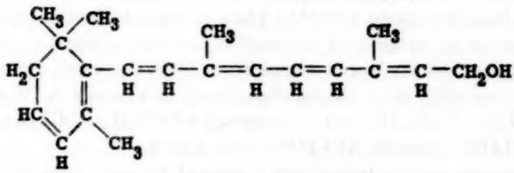
Recently it has been shown that retinene reacts also with sulfhydryl compounds, as do a number of other aldehydes. With cysteine and glutathione it forms addition compounds. These products possess a λ_{max} at about 340 mμ and do not change in spectrum with pH (113).

Vitamin A$_2$ and Retinene$_2$

The first correct characterizations of vitamin A$_2$ and retinene$_2$, and the first evidence of their vitamin function, appeared in a description of the biochemistry of the porphyropsin visual system in freshwater fishes [Wald (94)]. The term vitamin A$_2$ was not used in this description but was introduced shortly afterward on the basis of the visual observations (34, 61). A few months earlier, Lederer & Rosanova (60) had described "an abnormal reaction" in the antimony chloride test with liver oils of freshwater fishes. After the appearance of the visual observations, it was clear that this also had involved vitamin A$_2$ (cf. 98).

Since its discovery, vitamin A$_2$ has had a remarkably tortuous history. There is no longer any need to dwell upon this, for the status of this substance has finally been clarified with its purification and the preparation of crystalline derivatives by Shantz (80); the determination of its biological activity by Shantz & Brinkman (81); and the total synthesis of vitamin A$_2$ and retinene$_2$ by Jones and his associates in Manchester (35, 36). It is now

clear that vitamin A_2 is a dehydrovitamin A, with a second double bond in the ring. Such a structure had been suggested by Gray & Cawley (40) but was later rejected for what seemed to be substantial reasons. It was again brought forward by Morton, Salah & Stubbs (70) and defended against strong opposition. Time has proved it to be right.



Vitamin A_2, $C_{19}H_{25}CH_2OH$

Retinene$_2$, like retinene, is easily prepared by chromatographic oxidation of the corresponding vitamin A on solid manganese dioxide (70, 107). We have found it best in this process to use only about half as much manganese dioxide to oxidize a given quantity of vitamin A_2, as is used to oxidize vitamin A. The preparation and properties of crystalline retinene$_2$ and some of its derivatives are discussed by Cama *et al.* (20, 21, 22).[6] Retinene$_2$ exhibits many of the properties already described for retinene$_1$. It forms amino complexes which change color with pH as does the product of bleaching porphyropsin in solution (20, 98), and with hydroxylamine it forms a comparable oxime.

The Retinene Reductase–Alcohol Dehydrogenase System

The bleaching of a purified solution of rhodopsin ends in a mixture of retinene and opsin, in part loosely coupled to each other ("indicator yellow"). In the retina, however, retinene is reduced to vitamin A.

Wald (96) had observed that fresh solutions of frog rhodopsin, prepared from retinas which had not been treated beforehand with alum, bleach further than do the same solutions after a period of aging. This is one of the dark reactions that follow the exposure of rhodopsin to light, and corresponds to the "*Nachbleichung*" described earlier by Hosoya (50). Bliss (10) found this change to be associated with the conversion of retinene to vitamin A.

[6] As these authors indicate, there remains a discrepancy between the antimony chloride tests reported for natural retinene$_2$ (λ_{max} 705 mμ) (94, 98) and that prepared synthetically or by the oxidation of vitamin A_2 on manganese dioxide (λ_{max} 730–740 mμ, gradually moving toward lower wavelengths). We have recently confirmed the 730 mμ maximum, however, in the antimony chloride test with natural retinene$_2$, extracted from freshwater fish retinas. The earlier report that it lay at 705 mμ was based upon faulty recordings by a spectrophotometer that was not intended for use beyond 700 mμ.

Soon afterward it was found that a suspension of rod outer segments or a washed brei of whole retinas reduces retinene to vitamin A only if supplemented with a heat-stable factor, present in water extracts of retina and in boiled muscle juice. The factor was identified as dihydro-cozymase ($DPNH_2$).[2] For this one could substitute cozymase and hexose diphosphate; in the presence of the latter substrate, rod outer segments and washed retinal tissue can themselves reduce DPN.[2] The enzyme which catalyzes the reduction of retinene to vitamin A was called retinene reductase. The process simply transfers two hydrogen atoms from $DPNH_2$ to the aldehyde group of retinene, reducing it to the alcohol group of vitamin A [Wald & Hubbard (117)]: $C_{19}H_{27}HC{=}O$ (retinene)$+DPNH_2$ (dihydrocozymase) $=C_{19}H_{27}CH_2OH$ (vitamin A)$+$DPN (cozymase).

This enzyme system introduces a second vitamin into the chemistry of the rhodopsin system, for the functional component of DPN is nicotinamide, the anti-pellagra factor of the vitamin B complex. It appears here in the peculiar position of helping to regenerate vitamin A.

Retinal breis and digitonin extracts contain a nucleotidase which rapidly destroys cozymase. The coenzyme is protected from the action of this enzyme by the presence of free nicotinamide or α-tocopheryl phosphate. Such an attack upon cozymase accounts for the decline in the capacity of fresh solutions of rhodopsin to reduce retinene, remarked upon above. Their activity is restored to its original level on the addition of new cozymase (105).

The retinene reductase system can be assembled in solution from the following components: the enzyme protein or apoenzyme, extracted with buffer from homogenized retinas or rod outer segments; dihydro-cozymase; and as substrate, retinene dissolved in aqueous digitonin. When these components are mixed, the retinene is reduced almost quantitatively to vitamin A (105, 107).

The same enzyme system is equally effective in reducing retinene$_2$ to vitamin A_2. The enzyme protein, extracted from either a rhodopsin or a porphyropsin retina, works equally well upon either substrate (105, 107).

Bliss (12) pointed out that this enzyme might be the well known alcohol dehydrogenase, which, with DPN as coenzyme, catalyzes equilibria between aldehydes and alcohols in a wide variety of animal tissues (63). He reported that a crude preparation of alcohol dehydrogenase from rabbit livers catalyzes the equilibrium between retinene and vitamin A. This was confirmed (53) with the crystalline alcohol dehydrogenase isolated by Bonnichsen from horse liver (15). Incidentally, yeast alcohol dehydrogenase does not act upon retinene or vitamin A (13, 53). The enzyme present in frog retinas also readily oxidizes ethyl alcohol to acetaldehyde, as does the liver enzyme (W. Yudkin, cited in 109). There is no reason at present to distinguish the retinal enzyme from the alcohol dehydrogenase of other animal tissues. I shall refer to it hereafter by this more general designation.

The equilibrium of the alcohol dehydrogenase system normally lies far

over toward the side of reduction, toward the formation of alcohol. When this system has completed its work in the retina or *in vitro*, no observable amount of retinene ordinarily remains. It is common practice, however, to drive such systems in the oxidative direction by adding an aldehyde-trapping reagent which prevents the system from coming to equilibrium by removing the aldehyde as fast as it is formed. In the presence of such a reagent, bisulfite or cyanide (12) or hydroxylamine (118), the alcohol dehydrogenase system oxidizes vitamin A to retinene with yields of 40 to 50 per cent.

It should be understood that to displace a system from its normal point of equilibrium in this way requires that work be done. The work is done in this instance by the exergonic trapping reaction.

In a careful study published just before his death, Bliss (13) examined the equilibrium established by the alcohol dehydrogenase system between retinene and vitamin A. He found it to lie much further toward oxidation, toward retinene, than the ethanol-acetaldehyde equilibrium. According to his measurements, in equilibrium mixtures in which cozymase is half reduced, half oxidized, the ratio of vitamin A to retinene is about 300:1 at pH 6, 30:1 at pH 7, and 3:1 at pH 8. Relatively little energy is needed therefore to drive this equilibrium in the oxidative direction. This has turned out to be a factor of the highest importance for visual systems.

The Synthesis of Rhodopsin

Many years ago Kühne recognized that rhodopsin is synthesized in the retina in two ways: a rapid "anagenesis" from yellow precursors, which occurs to some degree in the isolated retina, and even in solution; and a slower "neogenesis" from colorless precursors, which Kühne could observe only in the intact eye, and which he believed requires the cooperation of the pigment epithelium (58, p. 311). These processes can now be identified with the synthesis of rhodopsin from the yellow retinene, and from the colorless vitamin A.

Hecht *et al.* (46) and Chase & Smith (24) confirmed Kühne's observation that rhodopsin regenerates slightly in solutions which have been bleached (i.e., to retinene and opsin) and replaced in the dark. The largest regeneration they reported was 15 per cent.

It has lately been shown that when rhodopsin solutions are supplemented generously with retinene, they regenerate as much as 85 per cent after bleaching. A colorless, carotenoid-free solution of opsin alone, on mixing with retinene and incubation in the dark at room temperature, forms large amounts of rhodopsin [Wald & Brown (112)].

No molecules other than retinene and opsin are required for this process. It is a spontaneous, i.e., an exergonic or energy-yielding, reaction. The bleaching of rhodopsin requires energy, supplied usually as light. Given the opportunity, removal of light, retinene and opsin re-unite spontaneously to regenerate the visual pigment (112). The rhodopsin obtained in such experiments possessed an absorption spectrum displaced several mμ toward shorter

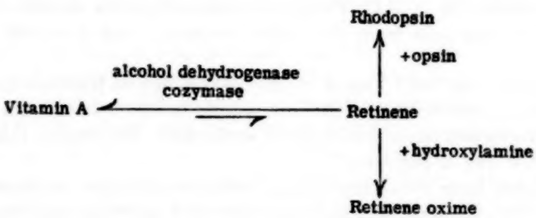
wavelengths (24, 112). It was what Collins & Morton had called in another connection "isorhodopsin." The cause of this difference is discussed below.

Opsin obtained from the porphyropsin retina of a freshwater fish unites with retinene to form rhodopsin, just as does that from frogs or cattle. Conversely, opsin obtained from either frogs or freshwater fishes, on incubation with retinene$_2$, yields a light-sensitive pigment intermediate in spectrum between rhodopsin and porphyropsin. It has been suggested that this may be "isoporphyropsin" (109, 112). In any case essentially the same results are obtained with opsins from freshwater fishes and land vertebrates. Like alcohol dehydrogenase, opsin appears to be freely interchangeable between the rhodopsin and porphyropsin systems.

The combination of retinene with opsin to form rhodopsin is inhibited by hydroxylamine (0.1 *M*) and by formaldehyde (0.7 *M*). The former competes with opsin for retinene; the latter competes with retinene for the active groups on opsin with which both aldehydes can combine (112).

The ease with which retinene and opsin unite to form rhodopsin suggested that if means were found to oxidize vitamin A to retinene, this might bring about the synthesis of rhodopsin from vitamin A. As already noted, however, the alcohol dehydrogenase equilibrium is in the reverse direction; it tends to reduce retinene rather than to oxidize vitamin A.

We have seen above that in the presence of an aldehyde-trapping reagent, the alcohol dehydrogenase system can be driven in the oxidative direction. Opsin is such a reagent, specifically a retinene-trapping reagent, prepared to perform this office in the retina. Just as hydroxylamine drives the oxidation of vitamin A to retinene by removing the latter in the form of its oxime, opsin should drive the same process by removing retinene in the form of rhodopsin (53):



Whatever the force of this argument, it had been recognized since Kühne that an isolated retina, though it would appear to contain all the components needed to synthesize rhodopsin in this way, seemed unable to do so. Careful extraction of whole frog retinas which had been bleached to colorlessness and then incubated in the dark showed, however, that they do in fact form a little rhodopsin, about 10 per cent as much as is formed during dark adaptation *in vivo*. Retinal homogenates behave similarly. Supplementing a retinal homogenate with cozymase doubles the yield of rhodopsin. Supplementing

it in addition with pigment epithelium homogenate doubles the yield again, bringing it to about 40 per cent (118).

One of the factors contributed by the pigment epithelium was shown to be vitamin A. The addition of vitamin A from any source to a retinal homogenate considerably increases the yield of rhodopsin. Another factor which increases the yield is the addition of the respiratory enzyme complex, the "succinoxidase" system, of pig heart. When all these factors were brought together, yields of rhodopsin were obtained as high as 60 per cent (53). Still higher yields have since been reported in breis of rat and frog retinas supplemented with vitamin A and other factors (26).

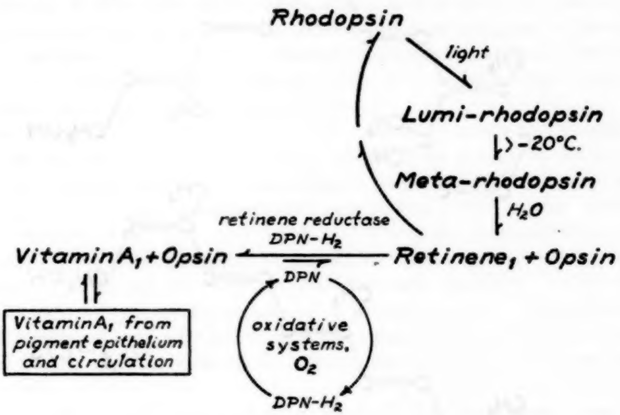


FIG. 1. Diagram of the rhodopsin system (53, 109).

It can be concluded that all factors which promote the oxidation of vitamin A to retinene aid in synthesizing rhodopsin—vitamin A itself, which tends to displace the alcohol dehydrogenase system toward retinene; cozymase, the oxidant of vitamin A; and respiratory enzymes and oxygen, which keep cozymase oxidized. The rhodopsin system can therefore be formulated as in Figure 1. What had originally been thought to be a special pathway for the synthesis of rhodopsin from vitamin A now appears to consist of the special conditions which promote the oxidation of vitamin A to retinene (53, 109).

If this view of the rhodopsin system is correct, it should be possible to bring the system together by mixing four substances: vitamin A, alcohol dehydrogenase, cozymase, and opsin. Such mixtures have been assembled in solution, using vitamin A from fish liver oils, crystalline alcohol dehydrogenase from horse liver, and yeast cozymase; only opsin needs to be taken from the retina. Such a mixture, placed in the dark, synthesizes rhodopsin. Brought out into the light, it bleaches; and replaced in the dark it makes

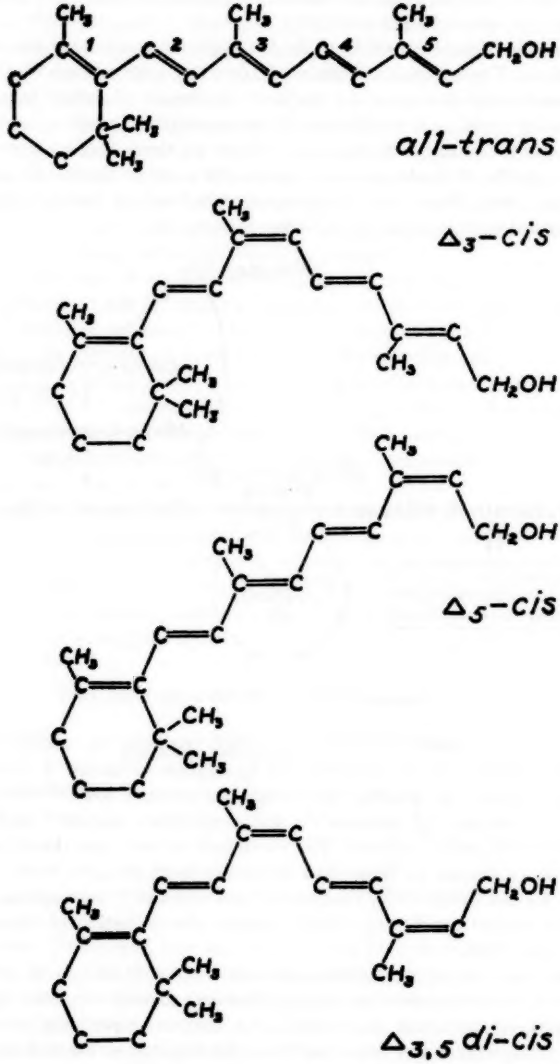


FIG. 2. *Cis-trans* isomers of vitamin A (54, 55).

more rhodopsin. This mixture of four substances carries out in solution all the reactions of the rhodopsin system (53).

Cis-trans Isomers of Vitamin A and Retinene in the Rhodopsin System

The four-component system just described was assembled originally using a fish liver oil concentrate as the source of vitamin A. When this experiment was repeated using crystalline vitamin A, almost no rhodopsin was synthesized. This discrepancy led to the following development [Hubbard & Wald (54, 55)].

Vitamin A, like other carotenoids, exists in a number of different configurations, *cis-trans* isomers of one another. Most of our present knowledge of *cis-trans* isomerization in carotenoids comes from the work of Zechmeister and his colleagues; in close association with these experiments Pauling has developed the theory of the subject (122).

According to present theory, in such structures as the carotenoids, *cis-trans* isomerization occurs readily only about those double bonds adjacent to methyl groups; elsewhere in the molecule it is hindered sterically. Hence vitamin A and retinene are expected to exist in four stable *cis-trans* modifications: all-*trans*, *3-cis*, *5-cis*, and 3,5-di-*cis*.[7] These are shown in Figure 2.

Ordinary crystalline vitamin A, as also the bulk of commercial synthetic vitamin A, is the all-*trans* isomer. Fish liver oils, however, are known to contain mixtures of the stereoisomers of vitamin A (75). The observation described above seems to mean, therefore, that rhodopsin can not be synthesized from all-*trans* vitamin A, but requires instead a *cis*-isomer present in liver oils.

It is well known from Zechmeister's work that any single geometrical isomer of a carotenoid is isomerized to a mixture of all possible isomers by exposure to light in the presence of a trace of iodine. When this is done with all-*trans* vitamin A, it becomes as effective a precursor of rhodopsin as fish liver oil concentrate.

The synthesis of rhodopsin therefore demands a specific *cis*-isomer of vitamin A. What is involved here primarily is the shape of the vitamin A molecule; this must fit appropriately the shape of a molecule with which it reacts, presumably a protein of the rhodopsin system. There are two such proteins, alcohol dehydrogenase and opsin. Alcohol dehydrogenase, however, has been found to exhibit very little stereochemical specificity, and that in the wrong direction. It is the combination of retinene with opsin that demands a specific *cis*-isomer of retinene.

Five isomers of retinene have now been prepared in crystalline condition: the all-*trans* isomer, first isolated by Ball *et al.* (4); neoretinenes *a* and *b*, isolated in the Harvard Laboratory (52, 55); and isoretinenes *a* and *b*, iso-

[7] As shown in Figure 2 the double bonds are numbered consecutively beginning with that present in the β-ionone ring. For a more complete discussion of terminology of *cis-trans* isomers see (122).

lated by the Organic Research Laboratory of Distillation Products Industries, Rochester, N. Y. Neoretinenes *a* and *b* and isoretinene *a* appear to be mono-*cis* forms, isoretinene *b* a di-*cis* retinene. The fact that there are five such isomers where only four were expected raises interesting problems for the theory of *cis-trans* isomerization in this class of compounds (52); but these have no place in the present review.

On incubation with opsin in the dark, all-*trans* retinene and neoretinene *a* yield no light-sensitive pigment. Neoretinene *b* forms rhodopsin (λ_{max} 500 mμ), indistinguishable from that extracted from the dark adapted retina. Isoretinene *a* yields a comparable pigment, with its absorption spectrum displaced about 13 mμ toward the violet (λ_{max} 487 mμ). It is suggested that this be called isorhodopsin, the term applied earlier to what appear now to have been mixtures of this pigment with rhodopsin. Isoretinene *b* in itself appears inactive, but on short irradiation or on standing in the dark at room temperature in the presence of opsin, it isomerizes preferentially to isoretinene *a*, which yields isorhodopsin.

All the isomers of retinene are freely interconvertible by gentle procedures. All of them, on simple exposure to light, go over eventually to the same equilibrium mixture of isomers. This, on incubation with opsin, yields mixtures of rhodopsin and isorhodopsin.

The immediate precursor of rhodopsin is therefore neoretinene *b*. All the syntheses *in vitro* described above had been performed with stereoisomeric mixtures of vitamin A or retinene and had yielded mixtures, in various proportions, of rhodopsin and isorhodopsin. Such mixtures of stereoisomers also included large amounts of inactive vitamin A or retinene, which remained unused at the end of the synthesis. When either neoretinene *b* or isoretinene *a* is incubated with opsin, the reaction is stoichiometric. In the presence of sufficient opsin, these isomers are converted entirely to rhodopsin or isorhodopsin, and no retinene remains at the end of the experiment.

The synthesis of rhodopsin or isorhodopsin follows a bimolecular course, when the appropriate isomer of retinene is mixed with opsin in approximately the proportions in which they combine. This is as though one molecule of retinene were reacting with one molecule or one combining center of opsin (55). When, as in the experiments of Chase & Smith (24), a large excess of opsin is present, the reaction simplifies to first order, the rate of synthesis being limited by the concentration of retinene alone (55).

In none of these syntheses, whether in solution from vitamin A or retinene (55), or in a brei of rat eye tissues (26) has there been any suggestion of such S-shaped regeneration curves as were observed under special conditions by Zewi (123) and regularly by Peskin (73) in living frogs.

Though isomerates of vitamin A or retinene yield mixtures of rhodopsin and isorhodopsin *in vitro*, the retina of a dark adapted animal contains rhodopsin alone. In this sense isorhodopsin is an artefact. Whatever processes supply vitamin A for rhodopsin formation *in vivo* must act selectively to single out neovitamin A*b* or to exclude isovitamin A*a*.

Retinene, which enters the synthesis of rhodopsin as neoretinene *b*, emerges from the bleaching of rhodopsin all-*trans*. It must be re-isomerized to the active configuration before it can regenerate rhodopsin.

Some years ago Chase (23) and Chase & Smith (24) reported that rhodopsin bleached with light containing blue and violet components of the spectrum regenerates slightly on incubation in the dark, but rhodopsin bleached in yellow light does not regenerate at all. They noted also that after bleaching rhodopsin in yellow light, exposure of the product to blue light, in addition to stimulating some regeneration, caused a little further bleaching.

The nature of these processes is now clear. Retinene is isomerized by light, but only by the blue, violet, or ultraviolet light which it can absorb. Yellow light, which it can not absorb, has of course no effect upon it. Yellow light can bleach rhodopsin but can not go on to isomerize the all-*trans* retinene which results. This in turn can not regenerate rhodopsin. On exposure of the all-*trans* retinene to blue or violet light it isomerizes, a change accompanied by a fall of extinction and a shift of λ_{max} about 5 mμ toward shorter wavelengths. This is the source of the extra bleaching which Chase & Smith observed in blue light. It constitutes a second photochemical step in the rhodopsin system. The mixture of retinene isomers to which it gives rise *in vitro* includes neoretinene *b* and isoretinene *a*, from which a mixture of rhodopsin and isorhodopsin can be regnerated.

Retinene therefore enters rhodopsin in one configuration and emerges in another. A cycle of stereoisomerization is an intrinsic component of the rhodopsin system. This can be formulated as follows:

$$\begin{tikzcd}
& \textbf{Rhodopsin} \arrow[dr, "\textbf{light}"] & \\
\textbf{Neoretinene b + opsin} \arrow[ur] \arrow[rr, leftrightarrow] \arrow[d, shift left] \arrow[drr, phantom, "\textbf{(alcohol dehydrogenase, cozymase)}"] & & \textbf{All - trans retinene + opsin} \arrow[d, shift left] \\
\textbf{Neovitamin Ab} \arrow[u, shift left] \arrow[rr, leftrightarrow] & & \textbf{All - trans vitamin A} \arrow[u, shift left]
\end{tikzcd}$$

The all-*trans* retinene which results from bleaching must be isomerized to neoretinene *b* before it can regenerate rhodopsin. Before this process has been completed, some all-*trans* retinene has been reduced to all-*trans* vitamin A, and this in turn must be isomerized to neovitamin A*b* before it can re-enter the rhodopsin system.

Some isomerization of retinene might be accomplished by light in the eye. There is also some, as yet unsatisfactory, evidence for the existence in the eye tissues of an enzyme which catalyzes the isomerization of vitamin A or retinene. This might explain the observation that water-soluble factors, apparently protein in nature, can be extracted from the combined pigment epithelium and choroid layer of the frog eye, which promote the synthesis of rhodopsin from retinene (14) and vitamin A (53). It may explain also a recent report that frog and rat eye tissues can use crystalline synthetic vitamin A,

the all-*trans* isomer, in synthesizing rhodopsin *in vitro* (26). The presence of such a retinene or vitamin A isomerase in the eye tissues would greatly aid the economy of the visual processes.

Beyond these possibilities, however, the eye must depend upon the blood circulation for new supplies of neovitamin A*b*. It was shown some years ago that during exposure of the eye to light, most of the vitamin A released in the retina escapes into the blood circulation and is re-captured during dark adaptation by being bound in nondiffusible form in rhodopsin (92). It is now apparent that these exchanges are stereochemically selective. The vitamin A which leaves the retina is primarily the all-*trans* isomer formed by the bleaching of rhodopsin; while that which is taken up from the circulation in synthesizing rhodopsin is exclusively neovitamin A*b*. These exchanges keep the visual processes in intimate connection with the metabolism and storage of vitamin A throughout the organism, and with an external factor, its supply in the diet.

The Role of Sulfhydryl Groups

Recently it has been shown that the synthesis of rhodopsin from retinene and opsin depends upon sulfhydryl (—SH) groups in opsin (113). These are not affected by monoiodoacetate, but on poisoning them with *p*-chloromercuribenzoate (10^{-4} M) rhodopsin synthesis is entirely blocked. This inhibition can be reversed in part by adding cysteine or glutathione to the reaction mixture.

Conversely, on bleaching rhodopsin, new —SH groups are liberated, two to three such groups for each retinene molecule that appears. This result is obtained with rhodopsin preparations from frogs, cattle, and squid. It seems probable, therefore, that in rhodopsin the carotenoid prosthetic group is bound to opsin in part through sulfur linkages (113).

These experiments have another interest. Sulhfydryl groups were measured by the amperometric silver titration of Kolthoff & Harris (57). In this arrangement the presence of free silver ions leads to a flow of current proportional to their concentration. Sulfhydryl groups, by binding silver ions, prevent the flow of current and can therefore be titrated. Such a titration can be performed in dim red light with a solution of rhodopsin. At its close, the addition of a small excess of silver ions leads to a flow of current. If at this point the rhodopsin is bleached by turning on a white light, the resulting liberation of new sulfhydryl groups causes a fall of current. By this means the bleaching of rhodopsin in solution yields directly an electrical variation. It has been suggested that this provides a significant model for the electrical changes which accompany the excitation of the retina by light, and indeed all nervous excitation (113).

It has been reported that intravenous injection of monoiodoacetate into cats and rabbits abolishes the electrical responses to light from the optic nerve and visual cortex, and greatly impairs or abolishes the retinal potential, all within a matter of several minutes (71). Some evidence is offered that the

principal effect is to posion glycolysis: yet there were indications of more direct effects upon visual structures in the degeneration of rods, cones, and pigment epithelium cells reported to follow this treatment (72). Possibly these changes are associated with the role of sulhydryl compounds in the retina. It should be noted however that monoiodoacetate, unlike *p*-chloromercuribenzoate, does not poison opsin or alcohol dehydrogenase. Furthermore, the concentrations of monoiodoacetate used in these experiments (15 to 25 mg. per kg.) seem very low for poisoning retinal glycolysis within the times indicated.

The Structure of the Rhodopsin Chromophore

What needs most to be explained here is the large change in color which accompanies rhodopsin formation. Retinene, with λ_{max} about 385 mμ, on uniting with opsin attains a λ_{max} of 500 mμ, a shift of spectrum toward the red of 115 mμ.

Originally it was suggested that this might be achieved by uniting two molecules of retinene end-to-end so that they would form a single conjugated system (106). This in itself, however, even if it included a new double bond at the point of union, should bring the λ_{max} only to about 480 mμ, as in such a molecule as β-carotene. Something more would be needed to bring it to 500 mμ.

Collins & Morton (29) have proposed a structure for the rhodopsin chromophore, based upon the union of two retinene molecules through a central nitrogen atom, originally from an amino group. With the demonstration, however, that the linkage of retinene to opsin in rhodopsin involves sulfhydryl groups, the amino group no longer holds the special position earlier accorded it.

Other considerations now make it seem more likely that the chromophore of rhodopsin is formed from one rather than two molecules of retinene:

(*a*) Much larger shifts of spectrum than are involved in rhodopsin formation accompany the treatment of vitamin A and retinene with antimony chloride or strong mineral acids. There is no suggestion that in these cases more than a single molecule of retinene is concerned. Incidentally, a number of such complexes are highly sensitive to light (5, 104).

(*b*) Retinene$_2$ possesses one more conjugated double bond than retinene$_1$; in consequence its λ_{max} is shifted about 20 mμ toward the red. If two retinene or retinene$_2$ molecules were joined end-to-end to form the chromophores of rhodopsin and porphyropsin, we should expect the latter to differ by two conjugated double bonds, and their spectra consequently by about 40 mμ. Instead they differ as do vitamin A and A$_2$, or retinene and retinene$_2$, by about 20 mμ.

(*c*) When retinene and opsin are mixed in approximately combining proportions, the kinetics of rhodopsin synthesis is bimolecular, as though one molecule of retinene were reacting with one of opsin (55). When opsin is in large excess, the kinetics apparently becomes monomolecular, the rate of

synthesis depending upon the concentration of retinene alone (24, 55). If two molecules of retinene joined to form the rhodopsin chromophore, however, the kinetics should remain bimolecular however great the excess of opsin.

Iodopsin

The first light-sensitive pigment of cone vision, iodopsin, was extracted from the chicken retina together with rhodopsin from the rods (95). The extraction was accomplished in aqueous digitonin. No alum could be used, since this destroys iodopsin and leaves only rhodopsin in the extract. The iodopsin was identified by differential bleaching. Cone vision, and hence iodopsin, are sensitive to deep red light, to which rod vision and rhodopsin scarcely respond. When the solution of photopigments extracted from the chicken retina is exposed to deep red light, it bleaches in a special way. The extinction falls maximally at 565 to 575 mμ, depending upon the pH. This is the bleaching of iodopsin. After it is completed, exposure of the residue to white light results in a renewed bleaching, maximal at 500 to 510 mμ, characteristic of rhodopsin. These observations have been confirmed in detail by Bliss (8), who added to them also an action spectrum of iodopsin.

Some confusion has resulted in the literature from the claims of von Studnitz, which still continue (89), to have made measurements upon the cone light-sensitive pigment of a turtle retina as early as 1932 (86), and to have extracted light-sensitive pigment from this tissue with ether in 1937 (87). These experiments have been commented upon elsewhere (44, 101), and there is no need to review them again. More recently von Studnitz has reported the extraction of a wide array of light-sensitive color vision pigments from the retinas of man and other animals, with ether, chloroform, methyl and ethyl alcohol, and other fat solvents (88, 89). I shall have more to say of this below. After a careful examination of von Studnitz's papers, I find no credible evidence that he has at any time observed, much less extracted, a cone light-sensitive pigment. As for the report that such pigments can be extracted with ether, Hosoya *et al.* (51), Wald (101), and Bliss (8) have been unable to confirm it.

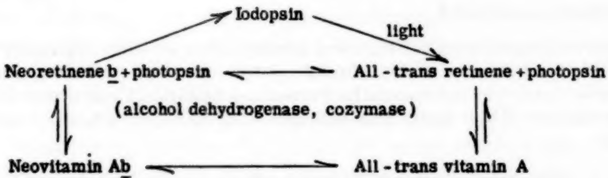
In experiments on human vitamin A deficiency, cone vision is found to deteriorate together with rod vision, and both recover together on administration of carotene or vitamin A (41, 47, 119). This, together with resemblances in the properties of rhodopsin and iodopsin in solution, made it seem likely that both pigments are closely related chemically, and that both might be derived from vitamin A (101). Bliss (9) showed that a product resembling retinene is released when chicken retinas are exposed to red light, under circumstances which should not have affected rhodopsin. We had performed similar experiments but felt them to be equivocal since the chicken retina in fact contains more rhodopsin than iodopsin, and the former pigment might have been the source of the retinene which is liberated (106).

These doubts have now been settled with an unequivocal demonstra-

tion that the bleaching of chicken iodopsin in solution yields retinene$_1$ as the product. Since chicken retinas also contain alcohol dehydrogenase, it can be assumed that subsequently retinene is reduced to vitamin A, in the cones as in the rods. Following the bleaching of iodopsin in red, i.e., non-isomerizing, light, there is no regeneration. The retinene which results is apparently the all-*trans* isomer. On addition of the same *cis*-isomer of retinene that regenerates rhodopsin, neoretinene *b*, to the bleached product, iodopsin regenerates quickly and completely. The carotenoid components of the rhodopsin and iodopsin systems are therefore identical (114).

The proteins, however, are different. To distinguish them, we can call the opsin of rod or scotopic vision *scotopsin*, that of cone or photopic vision *photopsin*. From light adapted chicken retinas one can extract into aqueous digitonin a colorless, carotenoid-free mixture of both proteins. On incubating this in the dark with neoretinene *b*, one obtains a mixture of rhodopsin and iodopsin indistinguishable from that extracted from the dark adapted chicken retina (114).

The iodopsin system therefore has the same composition and arrangement as the rhodopsin system, except for its protein. It can be formulated as follows:



Neoretinene *b* forms iodopsin in solution much more rapidly than it does rhodopsin. At room temperature the synthesis of iodopsin is complete within 4 to 5 minutes, while that of rhodopsin occupies about an hour. These observations provide a remarkable parallel with the well known fact that dark adaptation is much more rapid in cones than in rods (114).

Another basic element of visual physiology also comes directly out of the properties of rhodopsin and iodopsin. It has long been known that on transfer from dim to bright light, from rod to cone vision, the spectral sensitivity of the eye shifts toward the red. This is the Purkinje phenomenon. In the chicken, the spectral sensitivity of rod vision is maximal at about 520 mμ, that of cone vision at about 580 mμ (49). When proper allowance is made for distortion of these sensitivities by the carotenoid color filters of the cones and other factors, they appear to originate directly in the absorption spectra of rhodopsin and iodopsin (95).

The Problem of Color Vision

In the chicken, as also in a number of other birds and turtles, color differentiation is probably accomplished through a system of colored oil globules,

situated at the junctions between the inner and outer segments of the cones. There are three colors of such globules, and also colorless ones, making possible a system of three- or four-color vision employing the single photosensitive pigment, iodopsin. The pigments of the oil globules have been shown to be carotenoids: in the red globules, astaxanthin; in the orange, xanthophylls; in the pale yellow, a carotene (120). To these, a new carotenoid, galloxanthin, has recently been added (103).

The retinas of man and other mammals contain no such color filters, and it is supposed that here color differentiation depends upon the existence in the cones of a variety of light-sensitive pigments. It is an important task for the future to attempt to unravel this situation biochemically.

There has been much discussion in this realm of "modulators." This term was introduced by Granit some years ago to characterize retinal elements whose electrical responses to light display narrow sensitivity peaks. It was suggested that these involve receptors concerned with color differentiation (37a). The "modulators" seemed for a time to pose a direct biochemical problem, since they implied that light-sensitive pigments with similarly narrow absorption peaks might exist in the retina.

It is no longer clear that such an inference can be drawn. Granit (38) has recently concluded

> that the modulators need not represent primary colour-sensitive substances but can be, and in this particular work *are*, transformations of the spectral absorption curves of these substances by internuncial facilitation and inhibition. In particular, inhibition can be assumed to have reduced the ordinates along the regions where primary curves overlap.

That is, the narrow peaked modulators which Granit has observed electrophysiologically may represent pigments with quite different, and wider absorption spectra. As for the role of these units in color vision, which, though plausible, had never been demonstrated, Rushton (76) has recently found that responses of the type that revealed modulators in the cat retina, originally thought to come from single optic fibres, arise in fact from a rare, diffuse type of ganglion cell which appears "to collect impulses from a wide region of the retina, and to be ill adapted to discriminate form and colour."

I think it fair to conclude that at present the modulators are not in a position to serve either as guides to the properties of visual pigments, or to clarify the problem of color vision. The observation of Ball *et al.* (2, 5) that vitamin A and retinene on treatment with concentrated mineral acids yield spectra with sharp absorption peaks, thought to have some relation to modulators, is of considerable interest in showing the potentialities of these molecules for forming new chromophores, but appears to bear no more direct reference to color vision.

Since Helmholtz it has been recognized that the direct data of color vision are in accord with the view that the color receptors have broad rather than narrow spectral sensitivities, associated probably with light-sensitive

pigments which have broad rather than narrow absorption spectra (43, 83, 121).

We now have models of a number of ways in which such pigments, differing widely in the positions of their absorption spectra, can be obtained: (*a*) The carotenoid prosthetic group can be altered; so by uniting retinene and retinene$_2$ with scotopsin, one obtains rhodopsin and porphyropsin. (*b*) The same prosthetic group can be joined to different proteins; so by coupling neoretinene *b* with scotopsin and photopsin, one obtains rhodopsin and iodopsin. (*c*) Different *cis-trans* isomers of the prosthetic group can be joined with a single protein; so by combining neoretinene *b* and isoretinene *a* with scotopsin one obtains rhodopsin and isorhodopsin. All these ways of varying the absorption spectra of visual pigments could serve as the basis for constructing color vision systems.

The Number of Visual Pigments

The foregoing discussion has been based entirely upon three visual pigments: rhodopsin (λ_{max} in vertebrates, 500 ± 2 mμ); porphyropsin (λ_{max} 522 ± 2 mμ); and chicken iodopsin, the true λ_{max} of which is at 562 mμ.

The λ_{max} of squid rhodopsin lies outside the vertebrate range, at 490 mμ. It is this pigment, actually the corresponding meta-rhodopsin, that Bliss (11) suggested be called "cephalopsin." Since it possesses the same prosthetic group as rhodopsin and very closely related properties, we have preferred to call it squid rhodopsin (78).

Some years ago, Bayliss *et al.* (6) reported extracting from the eyes of marine fishes visual pigments, the spectral properties of which agree neither with rhodopsin nor porphyropsin. They spoke of these pigments as "new forms of visual purple." These observations have never been confirmed. Wald's measurements on a wide variety of marine fishes have disclosed only rhodopsin, except in the family of wrasse-fishes or Labridae, which possess mixtures of rhodopsin and porphyropsin, primarily the latter (90, 96, 102). Recently Kampa (56) has re-investigated the plaice, pollack, and gurnard, fishes which Bayliss *et al.* had reported to possess peculiar visual pigments, and found only rhodopsin.

Dartnall (31) has reported extracting from the retina of a freshwater fish, the tench, a "visual red" (λ_{max} 475 mμ) and a "visual blue-green" (λ_{max} 608 mμ). A second paper does not mention "visual blue-green," but adds "visual yellow 2" (λ_{max} 407 mμ) (32). This paper has been criticized by Collins & Morton (30). In a third paper Dartnall (33) has withdrawn "visual yellow 2" and has amended "visual red" to what is now called "visual pigment 467," from the position assigned to its λ_{max}. I take it that only this final pigment therefore needs to be considered.

The presence of "visual pigment 467" was revealed by bleaching an extract of tench retinas, first in red light, which bleached porphyropsin; and then in white light, which caused a slight further bleaching in the violet region. The difference spectrum associated with this second change was

maximal at 467 mμ, and was taken to indicate the new visual pigment. Such a procedure as this, however, involves the liberation of retinene$_2$ by the nonisomerizing red light, followed by its isomerization in white light. The isomerization of all-*trans* retinene or retinene$_2$ in white light induces qualitatively the same kind of change as Dartnall ascribed to "visual pigment 467" (cf. 55). In view of this it is not clear that one should postulate a new visual pigment, or if so, what its properties may be.

Von Studnitz (88), who does these things with a facility that one can only envy, has reported the extraction from human retinas with ether of three light-sensitive color vision pigments which bear a striking resemblance to the famous theoretical color vision primaries of Koenig & Dieterici [cf. Hecht (43)]. The source of the human retinas is not explained. Similar data obtained by the same means from a variety of other animals, frog, snake, chicken, guinea pig, are summarized in the same paper. Loevenich & von Studnitz (62) report extracting a fourth color vision pigment from the frog retina with "ether acidified with phosphoric acid." The authors point out that the existence of a fourth pigment need occasion no surprise, since direct absorption measurements on dark adapted retinas reveal at least 34 light-sensitive absorption peaks in the frog, and 21 in the chick. The authors caution that each of these peaks may not represent a different visual pigment; some of the pigments may possess more than one peak. For those who are interested in these experiments, I should add a further caution: read the papers.

I think one can summarize the situation at present as follows: in the rods of vertebrates there is substantial evidence for the existence of only two visual pigments, rhodopsin and porphyropsin. The distribution of these pigments has been explored to the point at which it begins to seem probable that vertebrate rods contain no others (102).

On the other hand, iodopsin marks only a beginning with the visual pigments of vertebrate cones. More pigments than this are needed, if only to provide the basis for color vision in mammals.[8]

In invertebrate eyes, the only visual pigment that is as yet well defined is squid rhodopsin. Here, however, it is altogether probable that further exploration will reveal a number of new visual pigments (cf. 7, 74).

[8] Perhaps the time has come to issue a warning against the uncritical assumption that if a tissue extract exhibits small changes in absorption spectrum after standing in the light, sometimes for hours, this is evidence of the presence of a visual pigment. It would be salutary to try such experiments with other organic solutions than eye tissue extracts. Tissue extracts are highly perishable. They undergo changes in the dark as well as in the light, and like all organic and much inorganic material, they are likely to change faster in the light. Using some of the methods lately invoked to discover visual pigments, one probably could have found them as well in muscle or liver extracts as in the eye. But why stop there? Beer changes in the light; that is why it is sold in brown bottles. I think it very probable that on careful examination one could find a "blue modulator" in beer.

The Distribution of Visual Systems

This has been reviewed in detail elsewhere (102, 111). The rods of marine fishes and land vertebrates characteristically contain the rhodopsin system alone. The only exception to this rule so far observed involves the marine wrasse-fishes, which possess mixtures of rhodopsin and porphyropsin in which the latter predominates. All freshwater fishes so far examined have porphyropsin alone. Euryhaline fishes, those which can exist as adults in either fresh water or the sea and so can migrate between both environments, possess either mixtures of both visual systems, in which that which goes with the spawning environment predominates, or exclusively the system that goes with the spawning environment. Thus the euryhaline salmonids, which spawn in fresh water, have predominantly porphyropsin; and the eel, which spawns in the sea, has predominantly rhodopsin (97, 98, 99).

A number of recent observations fall into this pattern. Collins & Morton (27) have found the haddock, a marine fish, to have rhodopsin (λ_{max} 500 mμ); the freshwater pike and perch, porphyropsin (λ_{max} 525, 524 mμ); while the char, a euryhaline salmonid, displayed such an intermediate spectrum (λ_{max} 514 mμ) as we have found to be characteristic of the mixtures of both pigments which occur in these forms. Similarly Kampa (56) has found rhodopsin in the marine plaice, pollack, and gurnard; porphyropsin (λ_{max} 520 mμ) in the freshwater tench; and an intermediate spectrum (λ_{max} 510 mμ) in a euryhaline salmonid, the trout.

The porphyropsin system, originally thought to be restricted to fresh water and certain euryhaline fishes, has been found to be distributed more widely. It predominates in the retina of the sea lamprey, a cyclostome, representative of the most primitive living class of vertebrates (100). The bullfrog possesses the porphyropsin system as a tadpole, and at metamorphosis transfers to the rhodopsin system. A similar metamorphosis of visual systems in the opposite direction was observed in another amphibian, the common newt, on going from its land-dwelling phase as a red eft to the mature water-phase adult. The mud puppy, *Necturus*, a permanently larval amphibian, apparently retains the porphyropsin system throughout its life (102, 111).

It has been suggested that this distribution of visual systems has evolutionary significance. The primitive freshwater vertebrates, like their contemporary descendants, the lampreys and freshwater fishes, probably possessed porphyropsin. The evolutionary migrations of vertebrates from fresh waters to the sea and to land both have involved the transfer to rhodopsin. Interpolated between freshwater and marine forms are the euryhaline fishes, which possess mixtures of both pigments. Similarly interpolated between freshwater and land forms are the amphibia, which seem to recapitulate in their metamorphoses the changes in visual chemistry which have accompanied vertebrate evolution (102, 111).

Invertebrates, both freshwater and marine, so far have been found to

possess only components of the rhodopsin system: vitamin A, retinene, and in the squid, rhodopsin (102, 111). The invertebrates which have been examined include representatives of both the phyla which have evolved well-formed eyes, the molluscs and arthropods. In these, as in the vertebrates, the evolution of visual systems appears to end in the utilization of vitamin A_1.

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FAT-SOLUBLE VITAMINS[1]

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Vitamin A

Chemistry.—Details on several new syntheses of vitamin A (1 to 4) and related compounds (5) have been published, and the synthesis of carotene-like compounds has been extended by Karrer & Eugster (6) to "dodecacapreno-β-carotene" which contains 12 isoprene units and 19 conjugated double bonds. The absorption maximum at 531 mμ was as expected, although the characteristic triply-banded spectrum of the carotenoids tended to lose distinctness as the number of double bonds increased.

New synthetic derivatives of vitamin A included analogues in which the phenyl group replaced the β-ionone ring of vitamin A acid or alcohol (7). Two forms of the acid appeared to be *cis-trans* isomers at the α-β double bond. None of the phenyl derivatives possessed vitamin A activity; the possibility of antivitamin activity still remains open. The corresponding phenyl analogue of carotene, 1,18-diphenyl-3,7,12,16-tetramethyl octadecanonaene, has been synthesized by Eugster, Garbers & Karrer (8). Several stereoisomers of the compound were obtained (9), including the 5,6:13,14 di *cis* form which contains a *cis* configuration at a position "forbidden," or at least regarded as very unlikely by Pauling (10).

On treatment with HCl and chloroform the epoxide of vitamin A was observed to undergo spectrophotometric changes (11) consistent with the formation of a furanoid like those in the carotenoid series. The furanoid showed weak vitamin A activity.

The transformation of natural colorless compounds into carotenoids has been achieved by Koe & Zechmeister (12) who treated phytoene (13) or phytofluorene with N-bromosuccinimide, the reagent used by Karrer & Rutschmann to convert lycopene into dehydrolycopene (14). Chromatography on $CaCO_3$ resulted in a series of colored compounds with the spectral characteristics of carotenoids containing 7, 9, 11, or 13 double bonds in partial *cis* configuration. Phytoene, in addition, yielded some phytofluorene.

It now appears that the retinenes probably exist in as many different forms as vitamin A itself. Neovitamin A esters were separated from concentrates of natural esters by chromatography on watered alumina and were found to resemble the all-*trans* ester spectroscopically except for the presence of the *cis*-peak at 250 mμ and a shift of the regular bands to 280 and 390 mμ. Treatment of neovitamin A alcohol with MnO_2 yielded neoretinene$_1$ (15). Cama *et al.* have made a series of preparations containing retinene$_2$ by

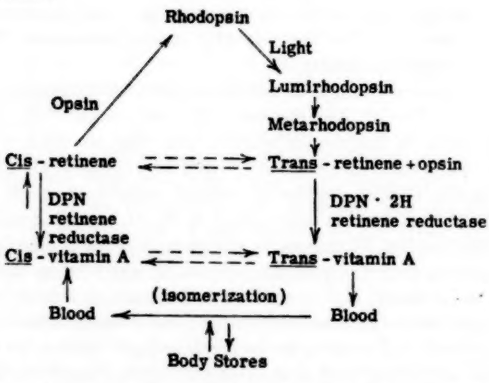
[1] The survey of the literature pertaining to this review was concluded in December, 1952.

treating unsaponifiable matter rich in vitamin A_2 with MnO_2. The purest product obtained after chromatography was an aldehyde, $C_{20}H_{26}O$, with characteristic absorption consistent with the assumption that the additional double bond is conjugated with the others. This is an argument against the lycopene-like structure proposed for vitamin A_2 (16, 17). However, Meunier (18) oxidized lycopene with MnO_2 and obtained a product with the spectroscopic properties of vitamin A_2, which, after reduction with lithium aluminum hydride promoted the growth of rats. The oxidation of β-carotene over MnO_2 in ether yielded a "retinene hydrate" which had growth promoting powers similar to vitamin A_1. Retinene$_2$ reacted with aliphatic amines under mild conditions (low concentration, room temperature, aqueous alcohol) to form spectroscopic analogues of acid and alcohol indicator yellow, in a manner like that previously reported for retinene$_1$ (19). The immediate reaction is assumed to be the formation of $RCH=NX$ which on acidification undergoes a marked shift in absorption with a possible conjugation of two C_{20} units.

Cama *et al.* have treated vitamin A_1 with aluminum tertiary butoxide and diethyl ketone and obtained a "dehydrated retinene$_1$," $C_{20}H_{26}O$, which differed from retinene$_2$ (20). It was reducible both *in vivo* and *in vitro* but failed to yield vitamin A_2.

The preparation of rhodopsin has been improved by the use of centrifugation in a sucrose solution in which a density gradient has been established (21). Cattle rods have the same density as 0.88 *M* sucrose.

Vision.—The simple visual cycle proposed by Wald in 1937 (22) has undergone substantial elaboration, and may now by summarized in the following scheme:



The bleaching of rhodopsin apparently takes place in several stages, which are detectable by spectrophotometric means when rhodopsin is irradiated at very low temperatures or in dry gelatin films. At −40°C the first product, lumi-rhodopsin, differs only slightly in color and spectrum from that of

rhodopsin itself, shifting about 5 mμ toward the shorter wave lengths and increasing somewhat in intensity. In the dark, at temperatures above −20°C. in solution, or at room temperature in the dry state, the next product is meta-rhodopsin involving a further shift in the spectrum toward the blue. At moderate temperatures or in the presence of water, there is a marked change in spectrum, and retinene is formed. The speed of vision is such that only the initial step, the formation of lumi-rhodopsin, is regarded as participating in the excitation of the optic nerve (23, 24). In the bleaching of rhodopsin two sulfhydryl groups are liberated for each retinene molecule that appears, and the formation of rhodopsin also seems to depend upon the availability of free sulfhydryl; treatment of opsin with *p*-chloromercuribenzoate prevents rhodopsin formation (25). The inhibitor did not react with retinene$_1$ nor did it destroy rhodopsin. It is quite possible that the liberation of sulfhydryl groups is the step by which electrical energy is released by the rods to the nervous system, since both the weak acidity and the reducing potential of this grouping can be made the basis of an electrical effect (26).

Rhodopsin is formed *in vitro* in the system: opsin, alcohol dehydrogenase, coenzyme I, and vitamin A, provided the proper stereoisomer of vitamin A is present (27, 28). Concentrates from fish oils were effective, whereas crystalline synthetic vitamin A failed to react. The usual preparations of crystalline vitamin A represent the stable all-*trans* isomer, whereas fish livers oil contain mixtures of all-*trans* and various *cis* isomers (29, 30). Treatment of the synthetic vitamin A with iodine and light resulted in an isomerized product which served as a precursor of rhodopsin, and an inactive preparation of neovitamin A (5-*cis*) likewise became effective after isomerization. The most probable precursor is thought to be 3-*cis* vitamin A. The stereospecificity of the system does not depend upon the alcohol dehydrogenase, which reacts with many different primary alcohols, but rather upon the opsin. Retinenes prepared from vitamin A by passage through a column of MnO_2 retain the configuration of the original vitamin, and their ability to unite with opsin depends on this configuration. The ability to remain united may also be stereo-dependant. Retinene prepared in this way from liver oil vitamin A readily formed rhodopsin, whereas the retinenes prepared from crystalline vitamin A or from neovitamin A were almost entirely inactive in this synthesis. Special precautions were found to be necessary in these studies, since the retinenes readily isomerize in the presence of light without the presence of the usual iodine catalyst.

In their most recent study Hubbard & Wald (26) compared the activities of five stereoisomers of vitamin A as well as the corresponding five retinenes as possible precursors of rhodopsin. Only one of the retinenes, now designated as neoretinene b, yielded natural rhodopsin, with absorption maximum, λ max. at 500 mμ. An uncharacterized isomer, isoretinene a, yielded an isorhodopsin, λ max. at 487 mμ. The others were inactive, but one of them was easily converted into isoretinene a and therefore also yielded iso-

rhodopsin. All forms of retinene were readily isomerized into an equilibrium mixture by exposure to blue or violet light whereas orange or yellow light, being unabsorbed, failed to affect them. The proper form of retinene combined with opsin in a 1:1 molar ratio.

On exposure to light both rhodopsin and isorhodopsin were converted into all-*trans* retinene, and somewhat more slowly into all-*trans* vitamin A. The regeneration of these compounds into rhodopsin failed until some isomerization had taken place. Wald therefore suggests that isomerization is an essential step in the vitamin A cycle. While some isomerization could conceivably take place under the influence of light in the retina itself, it is more probable that the all-*trans* vitamin A released into the general body supply is isomerized thermally at body temperature. Since such isomerization is a slow process, the operation of the cycle would be aided by the existence of a vitamin A or retinene isomerase.

Methods.—The problem of accurately determining vitamin A and carotene continues to attract attention, and the results of several collaborative studies have appeared (31 to 34), as well as special studies adapted to margarine (35 to 39), feces (40), blood (41) alfalfa (42 to 45), plant materials (46, 47), and low potency fish oils (48). "Butter yellow no. 4" has been proposed as a test substance in the standardization of the MgO-celite mixtures used in the analytical separation of carotene from vitamin A (49).

$SbCl_5$ has been shown to be useful in the determination of both vitamins A and D, and the amounts needed are very much less than those of $SbCl_3$. Brüeggemann *et al.* (50, 51) concluded that it is not $SbCl_3$ which reacts with vitamin A in the ordinary Carr-Price test, but rather the small amounts of $SbCl_5$ present in the saturated solutions of $SbCl_3$ employed. One mole of vitamin A was found to react stoichiometrically with one mole of $SbCl_5$; two moles of $SbCl_5$ reacted with a mole of carotene.

The proper correction for extraneous absorption in the spectrophotometric determination of vitamin A is still uncertain (52), and the biological equivalent for absorption continues to depend upon solvent, state of combination of vitamin A, and the ratio of neovitamin A to the all-*trans* isomer. Other substances in oils which may complicate the spectrophotometric determination include kitol, vitamin A_2, the anhydro vitamins A, and the epoxides (53).

The results of biological methods continue to vary with factors that influence the physiological effectiveness of the various forms of vitamin A or of its precursors. In the rat assay these include sex, length of the depletion time, and weight change in the latter stages of the depletion period (54). The diets currently employed in vitamin A assays are still essentially those used by Steenbock and others in their pioneer studies on carotene 30 years ago. These diets do not promote optimal growth when supplemented with vitamin A, and the addition of a water-soluble liver concentrate results in increased growth during the depletion period (55). The principle that the basal diet should contain adequate amounts of all essentials except that being measured

would seem to be as applicable to the growth assay for vitamin A_1 as to those for other vitamins. Biological responses to vitamin A and its precursors are reported to be sensitive to tocopherol (56), lecithin (57), to the nature of the vehicle used to dissolve the vitamin (58), to penicillin (59, 60), to alkyl hydroquinones (61), and to other carotenoids (62). Not all of these effects are specific, the response to lecithin may actually be attributable to the presence of carotenoids (63), and penicillin is only effective in certain individuals. Others have failed to observe any stimulation as a result of penicillin on diets limiting in vitamin A (64).

Stereoisomers.—Synthetic 15,15′monocis-β-carotene, also called "central monocis-β-carotene" has been shown to have 1/2 to 1/3 of the activity of all-*trans* β-carotene for both rats and chicks, the greater relative potency being evident at the lower levels of administration (65). Both the all-*trans* and the di *cis* isomers of 16,16′homo-β-carotene ($C_{42}H_{52}$) were 20 per cent as active as all trans-β-carotene (66).

Biosynthesis.—In an extension of their studies of carotene synthesis by *Phycomyces blakesleeanus*, Goodwin & Lijinsky (67) observed an increase of as much as 400 per cent in the synthesis of carotene when the medium contained L-leucine or L-valine, leucine being the more active. The possibility was suggested that the isoprenic residues might arise by the decarboxylation of leucine. Other substances such as asparagine promoted the growth of the mold without stimulating the synthesis of pigment. Periodic measurements of nitrogen assimilation and carotene production showed that most of the carotene was synthesized after nitrogen assimilation had ceased (68, 69). The mixture of carotenoids produced include α- and β-carotene, plus γ-carotene, δ-carotene, lycopene, phytofluorene, and other fluorescent substances (70). Most of these latter pigments account for only a small proportion of the total polyenes present. Glover, Goodwin & Lijinsky concluded that acetate is probably not a specific precursor of carotenoids, since mycelia produced less carotene after transfer to a medium containing acetate than to one containing an equivalent amount of glucose (71). C^{14} from labeled acetate was found in lipid, protein, carbohydrate, and CO_2. Others, however, have concluded that 69 per cent of the carbon of β-carotene comes from acetate (72). Several compounds related to the ionones did not exert much effect on pigment synthesis, but β-ionone doubled the amount of the carotene produced (73). Unknown factors in Difco yeast extract also increased pigmentation.

Physiology and Pathology

Bone abnormalities.—A fairly fundamental role of vitamin A in bone development is suggested by the abnormalities seen when vitamin A is deficient (74, 75) or in excess (76, 77). Wolbach & Hegsted (78) have extended these studies to young deficient chicks in which all sequences in skeletal growth were retarded before the growth of the bird was affected materially. In the chick, as in some mammals, changes in the nervous system appeared to be caused by pressure exerted on the central nervous system

by the improperly developed bones of the skull and vertebra. Chicks, like mammals, also showed skeletal changes in hypervitaminosis A, characterized by an acceleration of all the histological sequences in bone growth (79). These changes are so reproducible that Wolbach & Maddock (80) have proposed a method for evaluating the biological potency of a concentrate by determining the degree of skeletal abnormality in young growing animals fed excessive vitamin A.

Metabolic factors.—The digestibility of carotene in both cows and goats was shown to be increased by thyroxine and reduced by thiouracil (81), and these drugs also affected the vitamin A content of milk secreted (82). On feeding carotene after a period of depletion the rates of increase in the carotene and vitamin A content of the milk were enhanced by thyroxine and diminished by thiouracil. Thyroxine also seemed to have some effect upon the esterification of the vitamin, since the decrease in the proportion of ester observed when carotene was no longer fed was accentuated by the administration of thyroxine. In normal cow's milk only 6.9 per cent of the vitamin was present as the free alcohol. Neither thiouracil nor iodinated casein exerted any consistent effect upon the vitamin A in the blood, liver, or kidneys of depleted pigs fed carotene or vitamin A (83). The amounts of the drugs were those used in fattening the animals or in hastening development. Thiouracil was also without effect in another experiment with rats fed or injected with acueous dispersions of carotene (84). The end points were the amounts of vitamin A found in the serum, liver, and kidneys. The administration of estrogen was found to increase the level of vitamin A ester in the blood of immature pullets (85), and it has been suggested that the hormone may aid in moving vitamin A from the liver to the developing yolk.

Ascorbic acid.—The evidence for the postulated connection between vitamin A and ascorbic acid continues to remain inconclusive, but strong enough to prevent abandoning the idea completely. Thus, Morehouse, Guerrant & Dutcher (86) have shown that the ascorbic acid content of rat liver decreased progressively when 20,000 to 80,000 units of vitamin A were fed daily. This might be a nonspecific result of impaired liver function. In cattle vitamin A deficienty severe enough to cause an increase in spinal fluid pressure was accompanied by a decreased concentration of ascorbic acid in the plasma (87). The administration of ascorbic acid failed to relieve the deficiency symptoms although the response to vitamin A was good.

Miscellaneous effects.—Prolonged treatment of rats with 800 gm. of quinacrine hydrochloride (atabrine) per kg. of ration did not affect the requirement of the rat for vitamin A, but the ability of the liver to hold the vitamin was impaired, and the rate of absorption of carotene and vitamin A was diminished somewhat (88). The former effect is presumably an expression of functional liver damage to the drug (89, 90) and suggests the diminished retention of vitamin A observed in the presence of dibenzanthracene or ethyl alcohol.

Methyl cellulose did not decrease the absorption of vitamin A (91). Adverse effects of mineral oil have been ascribed to stereoisomerization of carotene as well as to direct interference with absorption (92). The ability of rats to withstand cold was improved somewhat by an increased intake of vitamin A (93); resistance to x-ray did not appear to be affected.

McCarthy & Cerecedo have succeeded in producing symptoms of vitamin A deficiency in young mice from deficient mothers fed a diet low in fat and vitamin E, and devoid of vitamin A (94).

Farm Animals

Cattle.—Goodwin & Wilson (95) showed the levels of carotene and vitamin in cattle blood to remain fairly constant while the animals were on grass from May to October. The levels dropped after parturition, when large amounts of both carotene and vitamin A were transferred to the colostrum; 95 per cent of the colostral vitamin A was esterified. Cattle blood, and that from other species, has also been fractionated by Ganguly *et al.* (96). Artificially dried hays were consumed by calves in greater quantities than field cured hays, such that enough carotene was furnished to meet the needs of the calf (97). The best utilization of carotene was observed when the hay was mixed with an equal proportion of starter mash (98).

Thomas, Jacobsen & Moore (99) suggest that factors other than intake may influence the level of vitamin A in the blood of the calf. Low fat diets containing skim milk tended to raise the level of the vitamins in the blood while high fat diets sometimes depressed the blood level. The plasma concentration of carotene reflected intake much more closely than the plasma level of vitamin A (100). Natural esters were observed to be absorbed in the upper two-thirds of the calf intestine, while the absorption of carotene took place in the middle third (101). Very little carotene was detected in the intestinal wall. Given orally an aqueous dispersion of vitamin A was 5 times as effective as an oily solution in increasing the vitamin A content of milk; injected intravenously the dispersion was 15 times as effective (102).

Swine.—The minimum requirement of young pigs for purified carotene ranged from 10 μg. daily to overcome deficiency symptoms to 25 μg. to restore blood levels of vitamin A and to provide some liver storage (103). Natural sources fed at equivalent carotene levels were effective in the following decreasing order: alfalfa meal and pellets, carotene in cottonseed oil, suncured alfalfa, and yellow corn (104).

Poultry.—The minimum amount of vitamin A required for the growth of chicks to eight weeks of age was found to be between 500 and 1,000 I.U. per pound of feed (105). Crystalline vitamin A appeared to be most efficiently utilized for the storage of vitamin A in the liver, whereas carotene was least efficient and cod liver oil was intermediate. Very high levels retarded the growth rate. An apparent increased requirement for vitamin A in hot weather (106) was attributed to a decreased intake of food rather to a specifically

altered requirement. This appears to be another illustration of the fact that within limits the needs for vitamin A tend to vary with body size rather than with the amount of food metabolized.

Symptoms of vitamin A deficiency observed in adult turkeys (107) were quite similar to those in chickens. They included white pustules about the head, watery eyes and the accumulation of caseous matter, and the deposition of ureates in the kidneys and other internal organs. Egg production and hatchability were reduced. Quail were shown to require 5,000 I.U. of vitamin A per pound of feed for an accumulation of liver stores, although growth and survival were good at 3,500 I.U. (108). Vitamin A furnished as cod liver oil appeared to be somewhat more effective than an equivalent number of I.U. of carotene. The percentage of vitamin A ester in the yolk increased during incubation, and by the eighteenth day the vitamin in the embryonic liver as well as in the unabsorbed yolk was largely the ester (109). Liver reserves of vitamin A were decreased in avian coccidiosis (110).

Stabilization in feeds.—Proposals for stabilizing vitamin A include embedding 0.5 to 1.0 μ particles of the vitamin in a matrix of gelatin and protecting by an external coating of gelatin. Superior availability in rats and farm animals is claimed (111). Solid stable concentrates of carotene would also be preferable to oils for incorporation with feeds. Carriers tested include cottonseed meal, soybean meal, and rice bran (112) in the presence of added antioxidants, but none of those tested was completely satisfactory.

Burns & Quackenbush (113) report dry vitamin A products to be more stable than oils during storage, and while each of commercial products tested showed some loss, all retained more than 50 per cent of their initial activity after six months at room temperature. Mixing with corn or soybean meal seemed to improve stability. The stability of carotene in alfalfa in loosely capped jars was improved by mixing the alfalfa with nine parts of certain common food ingredients (114). Ground yellow corn and wheat bran were better diluents than yeast, cottonseed meal, or oats. Dry skim milk, extracted soybean meal, and a mineral mixture were without effect.

Vitamin A in Man

Surveys of plasma values of carotene and vitamin A have been reported for 44 women on institutional diets (115) and for 95 children (116). Seasonal variations in carotene were greater than those for vitamin A, and the correlation between intake and blood level was also better for the pigment than for the vitamin. This is consistent with the known function of the liver in accumulating excesses of the vitamin and releasing them as needed. In another survey on school children a correlation was observed between the dietary intake of ascorbic acid, carotene, and vitamin A and the serum levels of these vitamins (117).

Hypervitaminosis A developed in a nine-month old child given 176 drops of oleum percomorphum daily instead of the eight drops recommended (118). The level of vitamin in the blood reached 1120 I.U./100 ml. instead

of the normal 50 I.U., and there was swelling and tenderness of the distal ends of the radii and ulnae. Complete remission was recorded 10 days after supplementation was discontinued. A three-year old girl who received one teaspoonful of the oil daily for a year (240,000 I.U.) developed anorexia, cracked lips, a pruritic rash, pain in the extremities and cortical hyperostoses (119). When the dosage was discontinued, there was improvement within three days, after three weeks she began to walk again, and after two months she was apparently normal.

Vitamin D

Methods.—In a suggested improvement in the line test the bone is washed in a series of solvents prior to staining with $AgNO_3$ (1). The procedure is said to minimize irregularities in staining. Certain errors in the iodine trichloride method of Green can be avoided by a proper preliminary treatment of the floridin used in chromatography (2). A modified line test without loss of the animal has been proposed by Snyder, Eisner & Steenbock (3), who fed vitamin D to rachitic rats and then injected P^{32} intraperitoneally. External counts were then taken on the wrists of the anesthetized rats. Within limits the number of counts varied with vitamin D intake.

Mode of action.—An enzymatic function for vitamin D has been suggested by Zetterström & Ljunggren (4) who studied the effect of phosphorylated vitamin D_2 (5) upon the action of alkaline phosphatases from kidneys, bones, and intestines. The rate of the enzymatic release of the phosphate from phenyl phosphate was approximately doubled in the presence of the phosphorylated vitamin. Vitamin D phosphate also increased the oxygen uptake of mitochondria (6), the effect being particularly marked in the early stages of the experiment. The vitamin also appeared to activate phosphatase activity toward glucose-6-phosphate. Cartier & Picard (7) have concluded that alkaline phosphatase is not involved in the early stages of embryonic ossification. Calcification *in vitro*, however, is extremely rapid in media low in inorganic phosphate but containing organic phosphate such as disodium monophenyl phosphate (8). The explanation offered is that phosphate is probably released in the slice under the influence of alkaline phosphatase.

Vitamin D has been associated with citrate metabolism, since it was observed that citrate relieves rickets under some conditions, and that urinary calculi on certain low-P diets are high in citrate. Harrison & Harrison (9) have observed a depression in the blood citrate levels of rachitic infants, and a rise on therapy. The urinary excretion of citrate also increased on therapy. Bellin & Steenbock (10) showed citraturia to be increased in rats by either physiological or excessive doses of vitamin D, and the effect was particularly evident on low-P diets. Vitamin D increased the pH of rat urine on two different rachitogenic diets (11) while decreasing the pH of the small intestine. Rats treated with vitamin D showed a higher rate of calcium (Ca^{45}) turnover than normal rats. Citrate improved the rachitic condition without affecting Ca metabolism (12).

Over-all effects.—In New Hampshire cockerels vitamin D increased the weight, length, volume, and ash of the long bones, the ossification of the sternum, and the growth of the bone in the heel (13). The growth of the cartilage was not affected. Bits of tibia from a day-old chick gained or lost Ca according to the degree of rickets in the bird in which they were implanted (14).

Improved digestion of protein and retention of nitrogen is reported in Holstein calves fed vitamin D (15). The deposition of gallium was found to be increased in rachitic rats, but the effect of vitamin D on the deposition of radiogallium did not appear to be sufficiently regular for assay purposes (16). The pigmentation of the wool of Hampshire lambs was abnormal on diets low in vitamins A and D. Vitamin A by itself was ineffective in correcting the condition, but the combination of vitamins A and D was successful (17).

Inorganic phosphate (P^{32}) was shown to be poorly absorbed from the gut of deficient chickens (18) and both vitamins D_2 and D_3 improved absorption. Pantothenic acid, niacin, folic acid, and cobalamin (vitamin B_{12}) appeared to be involved in P deposition in bone. Dietary fat is another factor that may exert an indirect effect on calcification. Steenbock & Bunkfeldt (19, 20) have shown that fat decreases the percentage of bone ash on a low-P, rachitogenic ration, but that this effect does not occur when the diet is adequate in phosphate. They suggest that fat increases the growth and P requirement of the soft tissues, which then "appropriate more than their usual share of the absorbed phosphorus."

Provitamin D_3, 7-dehydrocholesterol, appears to be formed in small amounts from cholesterol in the intestinal wall (21). The process is reversible and enzymatic. Another compound which might be involved in transformations between sterols is Δ^7-cholestenol, which occurs in very large amounts in the skin of rats and certain other animals (22, 23, 24). A substance with antivitanim D activity has been found in the unsaponifiable matter of hay (25). The factor diminishes the activity of vitamin D by about 1/3, and appears to act in the body after the absorption of vitamin D.

Vitamin E

Methods and chemistry.—A method (1) which can be carried out with only the simple equipment available in any laboratory involves the elimination of interfering materials by chromatography and floridin or florex previously treated with stannous chloride (2). A clear separation of α-, β-, γ-, and δ-tocopherol has been obtained by paper chromatography in which vaseline-treated filter paper is the stationary phase and 75 per cent ethanol the mobile phase (3, 4). Special procedures for the determination of tocopherol in plasma (5), serum (6) or feces (7) have been proposed. The oxidation of *dl-α*-tocopherol with ferric chloride in methyl alcohol yields four colored oils and a colorless wax which have been separated on a column of zinc carbonate (8). The products appear to be quinones.

Distribution.—Turkey liver contained more tocopherol than other organs, which in the order of decreasing concentration were found to be heart, gizzard, leg muscle, and breast (9). Increases in the tocopherol content of the diet increased the tocopherol content of the tissues. The amount of tocopherol in the meat decreased on storage. Cereals and pulses are reported to be good sources of tocopherol, and the amounts present are increased during germination (10). Feeding tocopherol supplements to cattle did not influence the production of milk or of butter fat, but the tocopherol content of the butter was increased, and the development of oxidized flavors decreased (11). The tocopherol content of milk decreased on winter rations. Analyses have been published on the amounts of vitamin E in many different types of prepared milks and of proprietary infant foods designed to resemble human milk in essential nutrients (12). Tocopherol is furnished to the infant by human colostrum, human milk, cow's milk or goat's milk in descending order. The amounts in proprietary baby foods were highly variable, and usually provided much less tocopherol than human colostrum.

Activity.—A series of esters of α-tocopheryl hydroquinone were compared for their ability to reduce creatine excretion in dystrophic rabbits (13). Given orally, only the succinate was as active as the free hydroquinone, but by intravenous injection the diphosphate and the diacetate also showed comparable activity although the triacetate was less active. The derivatives are recommended for parenteral use because of their purity, stability, and ready solubility. α-Tophocerol was found to be absorbed more rapidly by man than the acetate (14). Blood levels were used as a measure of absorption. Various polyhydroxy acids were shown to enhance the activity of α-tocopherol in stabilizing fats. The oxidation product of tocopherol, α-tocopheryl quinone, dismuted to tocopherol and a red *o*-quinone when refluxed with phosphoric, citric, or tartaric acid (15), This type of reaction may explain the prolonged effectiveness of tocopherol in the presence of the acids.

Deficiency symptoms.—Vitamin E deficiency in the lamb was characterized by stiffness, muscle dystrophy, low plasma tocopherol, high urinary creatine, susceptibility to pneumonia, and sudden death (16, 17). Lesions in the right ventricle of the heart were often observed. Histological changes included hyaline degeneration of the sarcoplasm and coagulation necrosis. Degeneration of the heart began in the endocardium and proceeded to the epicardium; the Purkinje fibers were not affected. α-Tocopherol cured the symptoms if given before the advanced stages were reached. A syndrome identical with "stiff-lamb disease" was observed in lambs fed tri-*o*-cresyl phosphate (18), and the compound hastened the development of symptoms of vitamin E deficiency in rats.

Some of the symptoms of vitamin E deficiency in male poultry may be secondary to changes in the pituitary gland. Glands from deficient birds contained about half as many active basophilic cells as normal, and assay

of the glands revealed a decrease in gonad-stimulating factors (19). White leghorns appeared to be more resistant to encephalomalacia than Rhode Island reds or Plymouth Rocks (20).

Blood changes.—The hemolytic action of dialuric acid on erythrocytes from tocopherol-deficient rats could be inhibited *in vitro* by tocopherol or by certain other fat-soluble antioxidants (21). *In vivo*, only tocopherol was effective. The hemolysis seemed to be associated with the formation of hydrogen peroxide in autoxidation. Hemolysis in hydrogen peroxide was observed in the blood of 23 premature infants fed evaporated cow's milk or mixtures derived from cow's milk, and the condition persisted unless tocopherol was fed (22, 23). Even large daily doses of tocopherol given to the mother during the last weeks of pregnancy failed to alter the hemolytic response of the new born. Another type of change reported in vitamin E deficiency is a marked increase in peripheral granulocytes in rabbits (24).

Metabolic effects.—The hepatic liver necrosis in rats fed a tocopherol-free *Torula* yeast responded better to cystine than to methionine (25), and an impaired conversion of methionine to cystine is thus suggested. Metabolic abnormalities in vitamin E deficiency include an increased synthesis of myoglobin without an actual increase in the tissues (26), a decrease in creatinine excretion along with an increase in creatine (27), an increase in the xanthine oxidase activity of rabbit liver (28), and an increase in the turnover rate of nucleic acids (29). The vitamin did not prevent liver necrosis due to CCl_4 (30), nor did it appear to be involved in the pituitary-adrenal response to acute physiological stress (31). Choline oxidase was unaltered in vitamin E deficiency (32).

Carotenoids and vitamin A.—Relatively large doses of α- and γ-tocopherol or of α-tocopheryl acetate diminished the amounts of hepatic vitamin A derived from β-carotene, but did not affect storage when vitamin A itself was fed (33). Interference with the absorption of carotene was assumed; the most effective "inhibitor," α-tocopheryl acetate, reached maximum concentration in the intestinal wall eight hours after injection, whereas this maximum was reached only five hours after the feeding of free α-tocopherol. The absorption of carotene is known to be slow. Another possible explanation is that excess tocopherol may interfere with the conversion of carotene to vitamin A.

The vitamin A and carotene content of New Zealand butter decreased on poor summer pasturage, and supplements of carotene were ineffective unless tocopherol was also included in the ration (34). Maximum responses were observed on daily doses of 1 gm. of tocopherol per cow.

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WATER-SOLUBLE VITAMINS[1,2]

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This review takes into account the literature that has appeared on each of the water-soluble vitamins between the time the particular subject was last considered in *Annual Review of Biochemistry* and December 31, 1952. In some cases, i.e., vitamin B_{12}, folic acid, pantothenic acid, and biotin, the review covers the year 1952; in other cases, it may include a consideration of the literature that has appeared over a period of several years.

The objective has been to discuss briefly and to call to the attention of the reader the most important pertinent activities, problems, and speculations and to include a bibliography that will allow him to obtain more detailed information with a minimum of search. No special effort has been made to establish priority. In cases in which a later paper was found to be more inclusive, it has been cited in preference to the earlier paper. The references cited represent less than half of those available and, therefore, are not complete. The growing knowledge of the chemical mechanisms by which the vitamins function and the interrelations of these mechanisms with other chemical processes of the living organism demands a broader and deeper knowledge of biochemistry and allied fields than has been required in the past for an understanding of the reports of current research in this field. In many instances, it will be necessary for the reader to consult previous reviews and textbooks of biochemistry as well as the cited references, for a satisfactory background and understanding of the particular subject. Space limitations and the broad and complex nature of the subject do not permit a presentation that is independent of other source material.

Vitamin B_{12} or Cyanocobalamin and Related Compounds

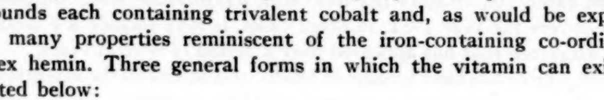
Although considerable research activity has continued on this subject since the review of last year [Welch & Nichol (1)], published reports, for the most part, expand and consolidate previous observations, ideas, and hypotheses. New developments have been few. Knowledge of the structure of

[1] The survey of the literature pertaining to this review was concluded in December, 1952.

[2] The following abbreviations are used: ACTH for adrenocorticotropic hormone; ADP for adenosinediphosphate; AMP for adenosinemonophosphate; ATP for adenosinetriphosphate; CF for citrovorum factor; CoA for coenzyme A; DCA for desoxycorticosterone acetate; DHA for dehydro-L-ascorbic acid; DKA for diketo-L-gulonic acid; DOC for desoxycorticosterone; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); FA for folic acid; FAD for flavin-adenine-dinucleotide; FMN for flavin mononucleotide; IF for intrinsic factor; NMN for nicotinamide mononucleotide; PGA for pteroylglutamic acid.

this complex molecule ($C_{61-64}H_{86-92}N_{13}O_{13}P$ Co, M about 1300) remains incomplete and although evidence continues to accumulate indicating the function of this vitamin in the metabolism of one-carbon intermediates, its primary biochemical role has not been revealed. The presence of this vitamin in a variety of forms and combinations, in natural and processed materials, continues to make the problems of analytical methods and nomenclature complex and difficult. In this review, the term, vitamin B_{12}, will be used in a generic sense to refer to this group of compounds. Specific compounds will be referred to by specific names, i.e., cyanocobalamin, etc.

Chemistry.—Vitamin B_{12} comprises a group of complex co-ordination compounds each containing trivalent cobalt and, as would be expected, shows many properties reminiscent of the iron-containing co-ordination complex hemin. Three general forms in which the vitamin can exist are indicated below:



I Cyanocobalamin | II Aquocobalamin | III Dicyanocobalamin

FIG. 1. Classes of Cobalamin.

Cyanocobalamin (I), a central member of this group of compounds, has a co-ordination number of six (bound to six groups) and a net charge of zero. The cyano group contributes one negative charge and satisfies one co-ordinate position; the cobalt^{+++} is co-ordinated to two other negatively charged groups and to three groups that carry no net charge. The structure of one of these groups is known to be 2′ or 3′-phosphoryl-1-α-D-ribofuranosyl-5,6-dimethylbenzimidazole (α-ribazole phosphate), which was isolated previously as an acid degradation product of cyanocobalamin and which has now been synthesized by Kaczka, *et al.* (2); the other groups are as yet unknown. Two moles of D_g -1-amino-2-propanol have been identified among the degradation products, and some evidence indicates that pyrrole-like nuclei may make up a part of the remaining unknown parts of the molecule [Folkers (3)]. Brink & Folkers (4) have described in detail the isolation and identification of 1-α-D-ribofuranosyl-5,6-dimethylbenzimidazole (α-ribazole), and Holly *et al.* (5) have published a synthesis of the 1-D-ribosides of 5,6-dimethylbenzimidazole. Davies *et al.* (6) have presented further evidence of the presence of co-ordination linkages in the vitamin B_{12} molecule by spectroscopic and other comparisons with synthetic benzimidazole-cobalt compounds. The presence of benzimidazole in the intact B_{12} molecule is supported also by new spectroscopic studies [Beaven & Holiday (7)].

Smith *et al.* (8) have studied the structures and stabilities of a series of cobalamins by the use of tracer techniques, absorption spectra, and partition coefficients. On the basis of these and other studies, the more general interpretation that there are three classes of cobalamins, neutral, basic, and acidic, has been offered (see Fig. 1). Cyanocobalamin (I), as a whole, is a neutral molecule. The cyanide ion can be replaced by other ions with strong co-ordinating tendencies to yield other neutral cobalamins, i.e., nitrite and thiocyanate give nitritocobalamin (vitamin B_{12c}) and thiocyanatocobalamin. These compounds are nonionic, comparatively stable, and obtainable in crystalline form. Photolysis and reduction with hydrogen (and a catalyst) or certain other reducing agents, displace the cyanide ion from cyanocobalamin to yield vitamin B_{12b} (B_{12a}). This compound seems to be an equilibrium mixture of hydroxocobalamin and aquocobalamin (II). The latter is probably the dominant form except in alkaline solution. This cation can form salts (ionic bonds) with strong acids, such as sulfuric and hydrochloric, and displays salt-like properties, i.e., treatment with barium acetate yields barium sulfate, etc. [Smith (9)]. Ellis & Petrow (10) suggest that this series of compounds be referred to as aquocobalamin sulfate, aquocobalamin chloride, etc., instead of as sulfatocobalamin and chlorocobalamin. Another member of this class of basic B_{12} vitamins is the substance, cobalichrome, in which NH_3 replaces the H_2O in aquocobalamin. This type of substitution is of interest because there may exist a series of cobalichromes, compounds in which amino acids, peptides, or proteins substitute for the H_2O in aquocobalamin. The vitamin B_{12} complex may exist in acid form (III) when it contains two similar or different univalent anions or one divalent anion, i.e., SO_3^{--}. This group of compounds is unstable and exists only in solution in the presence of an excess of the ions. Dicyanocobalamin, a purple cyanide adduct obtained in alkaline solutions in the presence of excess cyanide, is of this type. For details of absorption spectra, partition characteristics and exchange reactions, the reader is referred to the above reference (8). Most of the compounds mentioned previously are converted to cyanocobalamin upon treatment with cyanide. Conn & Wartman (11) have described the kinetics of this reaction. Absorption spectra and other physical constants for vitamin B_{12c}, and B_{12d} (B_{12b}) have been published by Smith *et al.* (12). Frost *et al.* (13) have shown that strong reducing agents such as cysteine, thioglycollate, and especially ascorbic acid can rapidly destroy aquocobalamin with the liberation of the cobalt. Cyanocobalamin is comparatively more stable under the above conditions.

It seems evident that vitamin B_{12} exists in natural and processed products as cyanocobalamin, aquocobalamin, and perhaps also as cobalichromes in which peptides are involved; furthermore, these forms can, in turn, be bound to proteins or, perhaps, to peptides through other bonds. In addition, they are in some instances associated with other vitamin B_{12}-like compounds (pseudovitamin B_{12}) in which the benzimidazole structure has been substituted by another nitrogeneous configuration. Hausmann & Mulli (14)

have reported the presence in fish solubles (juice pressed from autoclaved fish) of a microbiologically active cobalt-containing pigment which becomes hematopoietically active after treatment with potassium cyanide. The same investigators previously reported preparations from sheep dung, bacteria, inactive liver extracts, and beef muscle that became microbiologically and hematopoietically active after treatment with proteolytic enzymes. It was sugggested that vitamin B_{12} peptide conjugates are involved, although no further evidence was presented. It seems clear from the work of Denton *et al.* (15), and others that the previously observed enhancement of vitamin B_{12} activity obtained by extraction in the presence of cyanide is a result of at least two effects, namely, the conversion of other forms of vitamin B_{12}, i.e., B_{12b}, to the more stable cyanocobalamin and to the conversion of bound or otherwise inactive forms of the vitamin. The microbiologically active vitamin B_{12}, extractable from autoclaved egg yolk, was greatly increased in the presence of cyanide. Also, cyanide increased the recovery of added vitamin B_{12} from 75 to 95 per cent. Pfiffner *et al.* (16) and Dion *et al.* (17) have obtained three pairs of cobalt-containing pigments from the harvest broth of a rumen anaerobe that are presumably the cyano and corresponding aquo compounds. Two of these pairs are microbiologically active (lactobacilli) and the other pair is inactive. None of the compounds is hematopoietically active. In these so-called pseudovitamin B_{12} compounds, the benzimidazole structure appears to have been replaced by adenine. Vitamin B_{12f}, which is microbiologically active but biologically inactive for the rat and chick is probably a member of the pseudovitamin B_{12} series [Lewis *et al.* (18)]. This substance (or substances) has been found in the feces of a large variety of animals, including man. Likewise, it seems probable that the three factors (A, B, C) found by Ford *et al.* (19) in calf feces, belong to this series of compounds. Present evidence indicates that these pseudovitamin B_{12} compounds may be involved in the metabolism of intestinal flora rather than in the metabolism of the host. The relationship between these compounds produced in the gastrointestinal tract and those reported by Hausmann & Malli (14) is not clear.

Determination.—Previously available chemical or spectrophotometric methods of analysis for vitamin B_{12} could be used successfully only on moderately pure concentrates. Rudkin & Taylor (20) have published a method based on the difference in light absorption, at 583 mμ, of cyanocobalamin and the dicyanocobalamin formed upon the addition of excess cyanide. By a preliminary extraction with benzyl alcohol, which eliminates extraneous pigments and effects a concentration of the vitamin, the method can be applied to fermentation broths and crude concentrates. A reliability, speed, and accuracy superior to other methods is claimed and the sensitivity is in the same range as that of the microbiological methods. Boxer & Rickards (21) have modified their cyanide method to eliminate interfering substances found in some concentrates. Heathcote (22) has discussed the proposed U.S.P. XIV spectroscopic method (absorption 361 mμ) for the measurement

of purity of vitamin B_{12} (cyanocobalamin) and points out that considerable amounts of aquocobalamin (also hematologically active) can be present without detection, and suggests a simple, reproducible distribution measurement as an improvement in the method.

Microbiological methods, because of their sensitivity and apparent simplicity, have continued to be the most widely used means of measuring vitamin B_{12}. However, a large number of papers continue to appear indicating the great limitations of the available methods, suggesting improvements, and expressing the need for better methods [Ford (23); Krieger (24); Loy *et al.* (25); Robinson *et al.* (26); Lens *et al.* (27)]. Therefore, in order to minimize errors in interpretation, it is essential that workers reporting the results of vitamin B_{12} determinations include the fullest particulars of the method of extraction, assay, recovery, type of standard, etc. *Lactobacillus lactis*, *Lactobacillus leichmannii*, a mutant of *Bacterium coli*, and *Euglena gracilis* continue to be the most widely used organisms for this assay. With some products they give results in fair agreement when conditions are carefully standardized; with others the differences are sufficient to serve as a guide in the separation of the various forms of the vitamin and analogous compounds which occur. The marked discrepancies between results of biological and microbiological assays of vitamin B_{12} further emphasize the necessity of biological checks before interpreting the results of microbiological assays in terms of animal requirements. The selection and use of an assay method must depend upon the purpose for which the assay is to be used and the type of material being assayed. The reliability of the results will depend upon the critical sense and skill of the assayer as well as upon the inherent limitations of the method. The influence of oxygen and the presence of reducing substances in the media have been studied further by Lees & Tootill (28). The stabilizing effects of bisulfite and nitrite have been reported by Prier *et al.* (29), and the role of desoxyribosides, purines, and pyrimidines in replacing vitamin B_{12} for *L. leichmannii* has received further attention by Downing *et al.* (30). Zygmunt (31) concluded that the relative activities of vitamin B_{12} and its analogues are variable, depending on the culture media and assay method used. Robbins *et al.* (32) have examined the specificity of *E. gracilis* with respect to vitamin B_{12} and a variety of analogues, both natural and synthetic, and find that the qualitative response is similar to that of the other organisms in general use. *E. gracilis* reputedly is more sensitive than other assay organisms and is free from stimulation by desoxyribosides. The specificity of both qualitative separation and quantitative measurement of vitamin B_{12} has been increased by the use of chromatographic and ionophoretic methods [Patte (33); Doctor & Couch (34); Ericson *et al.* (35)]. Davis & Chow (36) have used the rapid and complete absorption of vitamin B_{12} by deficient, resting *L. leichmannii* as a means of concentrating the vitamin and eliminating interfering substances previous to assay. Williams *et al.* (37) have developed a simplified and rapid method of assay in which the area of growth on agar plates, result-

ing from the diffusion of the specimen previously absorbed on a paper disc, is measured. Frost *et al.* (38) and Cuthbertson & Thornton (39) have reported studies on rat assay methods. Scheid *et al.* (40) have compared the rat growth, liver storage, and microbiological methods on meats.

Intrinsic factor.—Available knowledge on the nature and mode of action of IF (intrinsic factor) has been reviewed at midyear (41). IF has neither been isolated nor identified, and the mechanism of its action is still obscure; however, some new knowledge and some new techniques, which will stimulate and aid further work, bring the answers to these questions a bit closer. Important experiments of Welch *et al.* (42) indicate that 70 to 90 per cent of Co^{60}, given in the form of isotopically labeled vitamin B_{12}, is excreted in the feces when given to patients with pernicious anemia in remission, but only 5 to 30 per cent is excreted when given with IF. It seems evident that enhanced absorption is at least one effect produced by IF. This technique for measuring IF provides a method far simpler than that of hematopoietic response. Burkholder (43) is attempting to purify the vitamin B_{12} "absorption factor" present in swine gastric mucosa, on the theory that pernicious anemia is a disease caused by removal of vitamin B_{12} from the patient's digestive tract by a greatly increased microbial flora under conditions of achlorhydria and lack of IF. A technique for measuring the inhibitory action of mucosa preparations on the absorption of vitamin B_{12} by bacteria isolated from the gastrointestinal tract is being used to follow the purification. A bacterial absorption technique has been employed by Chow & Davis (44) for screening substances for ability to bind radioactive vitamin B_{12}. In turn, the influence of these substances on the absorption of vitamin B_{12} from the gastrointestinal tract is being studied. Any conclusions concerning the identity of vitamin B_{12} bacterial absorption factors (of which there may be many) with IF must, of course, be demonstrated by clinical potency of the material. Spray (45) has shown that when neutralized human gastric juice is mixed with vitamin B_{12} and heated at 100° C., a considerable part of the vitamin remains in a form which can not serve as a growth factor for bacteria, whereas, the power of the juice to potentiate the hematopoietic action of vitamin B_{12} by mouth is lost. Glass *et al.* (46) have found that electrophoretically homogeneous preparations of gastric mucoprotein possess IF properties. However, as pointed out in last year's review (1), since far more potent preparations than these have been obtained by others, the presence of concomitant active substances in the mucoprotein remains a real possibility in explaining these findings. Rosenthal & Sarett (47), in developing a method for extraction and measurement of vitamin B_{12} activity of human serum with *L. leichmannii*, re-emphasize the fact that the vitamin occurs in a bound nondialyzable form in serum. The average vitamin B_{12} content of 24-hour postabsorptive sera was 0.20 mμg. per milliliter (range 0.08 to 0.42 mμg.).

Megaloblastic anemias.—The recent literature on the pathogenesis of megaloblastic anemias, the value of vitamin B_{12} in their treatment, and the

interrelationship of other factors (i.e., folic acid) which influence their production, course, and response, has been so well summarized in a number of recent reviews that little can be added here [Ungley (48); Nieweg *et al.* (49); Girdwood (50); (51)]. Conley *et al.* (52) summarize their experiences using vitamin B_{12} in the treatment of more than 100 patients with pernicious anemia, over a period of 40 months, as follows:

Parenterally administered B_{12} was as effective as refined liver extract in producing and maintaining clinical and hematologic remission. No evidence was found that patients with uncomplicated pernicious anemia need any therapy other than vitamin B_{12}. Orally administered vitamin B_{12} was effective in the treatment of pernicious anemia provided the oral dose was about 100 times the amount which was adequate when given parenterally. Until the adequacy of oral dosage schedule has been demonstrated, patients with pernicious anemia should receive parenteral therapy. An intramuscular injection of 45 μg. of vitamin B_{12} given every six weeks appears to be an adequate dose for satisfactory maintenance therapy and protected against hematologic and neurologic relapse. Vitamin B_{12} seems preferable to liver extract in the treatment of pernicious anemia because it causes less discomfort at the site of injection, does not give rise to untoward reactions [see Bedford (53)] and is less expensive than liver extract.

While there have been some suggestions by others [Reisner & Weiner (54)] that folic acid in addition to vitamin B_{12} gives an enhanced hematopoietic response in pernicious anemia, this needs further study before acceptance [Sanneman & Beard (55)].

Ungley (56) has given some current views on the possible etiology of pernicious anemia and describes the atrophic lesions of the stomach as being primary. He also points out that much evidence indicates that a toxic factor, which might arise because of lack of detoxification by the liver of certain bacterial products absorbed from the gastrointestinal tract, could explain many of the symptoms of the disease (including nerve degeneration) that are at present not explainable as a result of the erythropoietic effect of vitamin B_{12}. Recent advances concerning absorption and utilization are summarized. It is evident that free vitamin B_{12} does not support the maturation of marrow cultures until combined with a thermolabile substance that is present in gastric juice and perhaps also in the intestinal mucosa but not in circulating blood. The author suggests that the small intestine plays a larger part in the biochemistry of vitamin B_{12} than has been generally suspected.

Certain non-Addisonian megaloblastic anemias also respond to vitamin B_{12}, but there are others which do not. The megaloblastic anemias of pregnancy, infancy, and steatorrhea seem to be primarily folic acid deficiencies. While the anemia accompanying sprue responds often to vitamin B_{12} [Suárez (57)], some or many of the anemias in these groups may be mixed vitamin B_{12} and folic acid deficiencies. At the moment, there is no way of determining before treatment which factor may be lacking or whether both are missing. Experimental megaloblastic anemia in monkeys (thought to be

analogous to the megaloblastic anemia of infancy), produced by a low ascorbic acid diet, responds to folic acid but not to B_{12} [May *et al.* (58)]. Cartwright *et al.* (59) have produced an experimental megaloblastic anemia in swine maintained on a diet deficient in both vitamin B_{12} and folic acid. While the addition of either vitamin gave some response, both were necessary for complete recovery.

Further studies on administration of vitamin B_{12} supplements to premature and newborn infants and undernourished children failed to reveal acceleration in growth rate, as previously reported [(51); (60); Spies *et al.* (61); Finberg & Chow (62); Chinnock & Rosenberg (63)].

Animal nutrition.—It is well established that food of animal origin (meat, fish, milk, etc.) improves growth and performance of a variety of animals kept on vegetable rations because it contains vitamin B_{12}; also, that in many cases, further benefits arise from the addition of small amounts of certain antibiotics to the ration [Cuthbertson (64); Coates (65)]. Further evidence has appeared indicating in food of animal origin, the presence of factors in addition to vitamin B_{12} that are required by the chick and the turkey poult. The hatchability of eggs and the growth of chicks and poults from eggs from hens maintained on rations low in animal products are improved by the addition of fish solubles, meat scrap, etc. to a ration already containing vitamin B_{12} and antibiotics. Some progress has been made in concentrating these unknown factors from liver and whey [Savage *et al.* (66); Sunde *et al.* (67); Scott & Jensen (68); Menge & Combs (69)]. Some corn-soy and corn-cottonseed meal rations have been found limited in methionine and lysine content respectively, and, therefore, these amino acids must be added to such rations to obtain the full benefits of added B_{12} [Machlin *et al.* (70)]. There is a close correlation among the vitamin B_{12} content of the hen's ration, egg content, hatchability, and growth of the chick during the early weeks after hatching. Although vitamin B_{12} requirements of the hen may vary considerably, depending upon a number of factors, from 2 to 4 μg./kg. of ration produces normal hatchability, while 4 to 6 μg./kg. are required for good growth and viability of the chicks; 2.5 mμg./gm. of yolk were found adequate for normal hatchability and can be added directly to the embryo. Hens with high levels of vitamin B_{12} in the ration will yield hatchable eggs and normal chicks longer after removal of the vitamin [Yacowitz *et al.* (71); Petersen *et al.* (72); Milligan *et al.* (73); O'Neil (74); Wiese *et al.* (75)]. For normal growth, weanling pigs reguire 4 to 6 μg. of vitamin B_{12} per pound of a corn-soy meal ration containing antibiotics [Catron *et al.* (76)]. The mode of action of antibiotics as growth stimulants in animals presumably receiving adequate diets is not established although it is generally thought that the effects is on the gut flora. Recent evidence suggests that growth-stimulating effects of antibiotics on chicks, pigs, and rats receiving adequate diets may be a result of the elimination of apparent or inapparent, growth-depressant, infective conditions. The effects often noted in the absence of vitamin B_{12} and other vitamins apparently

result from changes in intestinal flora that increase the synthesis, decrease the rate of destruction of these essentials, or both [Cuthbertson (64); Blight *et al.* (77); Sheffy *et al.* (78)].

Absorption and excretion.—Further studies on the absorption, excretion, and tissue distribution of administered vitamin B_{12} have appeared, aided by the use of the Co^{60}-labeled vitamin. Vitamin B_{12} administered orally to various species of animal, including man, results in the excretion of the vitamin largely in the feces and to a significant extent in the urine if the dosage is large. Rosenblum *et al.* (79) have found that in the rat, of a 4 μg. dose of radioactive vitamin B_{12}, 81 per cent of the radioactive cobalt is in the feces, and most of it is in a form indicating that the vitamin B_{12} has been degraded. One per cent of the dose was excreted in the urine and the rest was distributed in various tissues. The same dosage given parenterally led to excretion of 50 per cent in the urine and 6 per cent in the feces. Sokoloff *et al.* (80) report that in man 53 to 68 per cent of vitamin B_{12}, administered parenterally in doses of 84 to 211 μg., was excreted in 18 hours, the percentage increasing with the size of the dose. Initial doses of 42 μg. each gave little excretion, but excretion became appreciable after a number of such doses. No significant differences in excretion of the vitamin were noticed between six normal and six pernicious anemia patients. Assuming that the difference between the dose administered and the microbiologically active vitamin B_{12} excreted in the urine represents the amount of the vitamin retained, Lang *et al.* (81) have found that from 19 to 55 μg. of dosages ranging from 20 to 75 μg., given intramuscularly, are retained by the normal male adult. In the rat, the lower the previous intake of vitamin B_{12}, the greater was the retention of a given dose [Chow (82)]. That the retained vitamin is rather firmly held by the tissues is indicated by the failure to "flush out" tissue-held radioactive vitamin B_{12} by subsequent large doses of the vitamin. Distribution studies on the rat and the chick show that the kidney, liver, and pancreas retain respectively the highest amounts of the vitamin and that most other tissues retain some [(79); Monroe *et al.* (83); Yacowitz *et al.* (84)]. Ellingson *et al.* (85) have found three to four times as much radioactivity appearing in the milk of lactating rats that received a dose of vitamin B_{12} containing Co^{60} subcutaneously than when the same dose is given orally. While the efficiency of absorption of vitamin B_{12} from the gastrointestinal tract appears to be not high, it is evidently adequate under normal conditions in both man and experimental animals.

Biochemistry.—Additional papers have appeared emphasizing the importance of vitamin B_{12} in the synthesis of methyl groups and perhaps to other steps in "methyl" metabolism. However, the details of the mechanisms involved remain incomplete. The growth response of vitamin B_{12} deficient chicks to methionine, homocystine, betaine, and vitamin B_{12} and to various combinations of these supplements has been reported by Jukes & Stokstad (86). Liener & Schultze (87) have done similar experiments on rats, observing, in addition to growth response, the capacity to synthesize N-methyl-

nicotinamide and to form creatine. Vitamin B_{12} plus homocystine gave a response similar to that of homocystine plus choline, or that of methionine, thus indicating its participation in the synthesis of methyl groups. However, the response to homocystine plus choline or betaine was always improved by the addition of vitamin B_{12}, thus suggesting that in some way vitamin B_{12} improves the effectiveness of choline as a methylating agent. Although choline alone would not support growth and was ineffective in the methylation of N-nicotinamide, it led to a creatinine excretion in the rat comparable with that of the other supplements. In the absence of vitamin B_{12}, formate was ineffective as a methyl source for the rat. Dubnoff (88) has reported that homocysteine, glutathione, or cysteine can partially replace the vitamin B_{12} requirements of a mutant *Escherichia coli* and that the further addition of dimethyl-β-propiothetin can replace the major B_{12} requirement for this organism. Choline and betaine, which are active as methyl sources in animals, were completely inactive in this case. Arnstein & Neuberger (89) and Stekol *et al.* (90) have presented rather clear evidence that vitamin B_{12} is involved in some manner in making available the α-carbon of glycine for the synthesis of both the ethanolamine and methyl groups of choline although the low incorporation of the C^{14}-isotope in choline, compared with that obtained with serine or formate, makes it seem likely that the conversion is indirect, perhaps through serine. Vitamin B_{12} appears to have no effect on the conversion of either serine or formate to choline. It is, therefore, likely that the vitamin functions in the step, glycine to serine. Such a mechanism would also explain the observation that vitamin B_{12} counteracts glycine toxicity in the chick.

Machlin *et al.* (91) have confirmed the observation that vitamin B_{12} counteracts the toxicity of glycine for the chick and find that folic acid is also of some value in this respect. Hsu & Combs (92) have studied the effect of the addition of a number of amino acids to the rations of chicks receiving low vitamin B_{12} diets. Four per cent levels of glycine or L-leucine, or 6 to 8 per cent zein (high content of leucine) inhibited growth; this was counteracted by oral or subcutaneous administration of vitamin B_{12}. L-Tyrosine and DL-aspartic acid fed at the same levels exerted similar growth-inhibitory effects; however, vitamin B_{12} was only partially effective in overcoming this inhibition. Excess DL-alanine, DL-methionine, or L-cystine also depressed growth even when vitamin B_{12} was present. L-Glutamic acid and DL-lysine exerted only a slight growth-inhibitory effect. It will be recalled that niacin is also effective in overcoming certain types of amino acid imbalance. Thus, it appears that a number of factors are involved in amino acid imbalance and its alleviation in the chick and probably in other animals as well. The blood amino and urea nitrogen are elevated in vitamin B_{12} deficiency and this elevation is accentuated by glycine, leucine, etc. Administration of vitamin B_{12} led to a decrease in these values and in many cases to an increase in blood uric acid [Hsu & Combs (93)]. The high incidence of mortality of newborn rats with symptoms of acute uremia, found associated with mothers

receiving rations high in soy oil meal, and which is prevented by addition of vitamin B_{12} to the maternal diet, is undoubtedly an analogous condition [Schultze *et al.* (94)]. It is interesting that after the loss of several litters in the above manner, the addition of vitamin B_{12} provides a corrective measure.

Antagonists.—A number of synthetic compounds, i.e., benzimidazoles and other compounds similar in structure to the degradation products of vitamin B_{12}, have been tested for their vitamin B_{12} activity, or vitamin B_{12} antagonist action (or both) on the rat and chick. While a number of these show some vitamin B_{12} activity, it is of a low order of potency [Cooperman *et al.* (95); Vijayaraghavan & Dunn (96); Lambooy & Haley (97)]. The oxidation of cyanocobalamin with hydrogen peroxide in acid solution produces an unidentified product that competitively inhibits the use of vitamin B_{12} by *L. leichmannii* but has no effect on three other organisms that do not require vitamin B_{12} as a nutrient [Beiler *et al.* (98)]. The product does not inhibit *E. gracilis*, a vitamin B_{12}-requiring organism [Villella & Abreu (99)]. References to other compounds, which appear to function as antimetabolites for a number of species of microorganisms, may be found in a recent review (100).

Cobalt deficiency.—In the light of recent reports [Marston & Lee (101); Anderson & Andrews (102); Hoekstra *et al.* (103)], it seems clear that the earlier failures of cobalt deficient sheep to respond to parenteral vitamin B_{12} were a result of inadequate dosage. Apparently this species' requirement for vitamin B_{12} is higher than would be anticipated from other experiences, but is easily met by bacterial synthesis in the rumen when there is adequate cobalt in the diet. This also may explain why sheep show cobalt deficiency so readily when the cobalt content of pasturage tends to be low, a condition which occurs in many parts of the world [Marston (104)]. Monroe *et al.* (105) have studied cobalt metabolism in sheep by the use of Co^{60} and find some suggestion that the adrenals and spleen can synthesize vitamin B_{12} from parenteral cobalt. This is interesting, if true, since bacterial synthesis is the only previously known primary source of this vitamin.

Pteroylglutamic Acid or Folic Acid, Citrovorum Factor or Folinic Acid, and Related Compounds

Last year's review by Welch & Nichol (1) and reviews by Jukes (106) and Girdwood (50) should be consulted for general background on this subject. This present brief review, for the most part, will consider only significant progress reported during the past year. The terms, citrovorum factor (CF) or folinic acid, will be applied to those substances, natural and synthetic, that support the rapid growth of *Leuconostoc citrovorum*, a property not shown by PGA (pteroylglutamic acid). *Streptococcus faecalis* and *Lactobacillus casei* respond similarly to PGA and CF. The term, folic acid (FA), will refer to those compounds, natural and synthetic, that support the growth of *S. faecalis* and *L. casei*. PGA will refer specifically to pteroylglutamic acid. In the literature, some authors have used the term, folic acid, as pro-

posed above while others have used the term synonymously with PGA. The intent of the authors must be determined in each instance.

Chemistry.—Roth *et al.* (107) have described in detail a synthesis of leucovorin (synthetic citrovorum factor, folinic acid-SF), a formyltetrahydropteroylglutamic acid, active for *L. citrovorum*, 0.15 mμg. of which is equivalent to one "unit" as defined by Sauberlich (107). Essential steps in the synthesis are: reduction of pteroylglutamic acid or 10-formylpteroylglutamic acid in formic acid solution over a platinum catalyst whereby two moles of hydrogen are absorbed; neutralization with aqueous sodium bicarbonate solution; adjustment with sodium hydroxide to pH 10 to 12, followed by heating; subsequent isolation of the barium or calcium salt by chromatographic methods; crystallization of the free acid at pH 3.5. Extensive studies [Cosulich *et al.* (108)] indicate that the synthetic product has the structure previously proposed and synthesized by Flynn *et al.* (109) and by Brockman *et al.* (110), 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid. The properties shown upon acid treatment (loss of activity for *L. citrovorum* but retention of activity for *S. faecalis*) are analogous to those shown by natural CF. During the synthesis by the above method, a new asymmetric center is produced at carbon atom-six. Since the L-glutamic acid moiety of the compound contains an optically active carbon, the product obtained consists of a mixture of diastereoisomers, *d*L and *l*L forms. Cosulich *et al.* (111) have obtained the *l*L isomer in what appears to be a pure form by crystallization of the calcium salts. The biological activity of this isomer for *L. citrovorum* and for *S. faecalis*, both before and after treatment with acid, appears to be the same or perhaps a bit higher than that for the natural CF (supplied by Keresztesy) and double that found for the mixed isomers. The *d*L isomer (not pure) showed low activity for both CF and FA. This would seem to establish the identity of the *l*L isomer of synthetic CF with the natural compound. Sauberlich (112) has prepared CF concentrates from spinach, urine, and liver and compared their properties with those of leucovorin. In general, the active material in the three concentrates appeared to be the same and analogous to the synthetic CF.

Broquist *et al.* (113) have compared the biological activity (growth) of leucovorin (synthetic CF) and PGA for *L. citrovorum*, *S. faecalis* 8043, *L. casei* 7469, the chick, and turkey and also the capacity of these compounds to reverse the toxicity of aminopterin. It was found that 0.15 mμg. of leucovorin as the anhydrous free acid is required per milliliter of culture medium to produce half-maximum growth of *L. citrovorum*. About 200,000 times as much PGA is required by this organism for comparable growth. About twice as much leucovorin as PGA is required for similar growth with *S. faecalis* and *L. casei*. In the chick and turkey, the leucovorin when injected is also approximately half as active as PGA in stimulating both growth and hematopoiesis. This difference in both cases is undoubtedly a result of the use of the isomeric mixture of which the *d*L form is inactive. However, oral PGA is more effective than oral leucovorin even taking into

account the isomer problem. Leucovorin is strikingly more effective than PGA in reversing the toxicity of aminopterin both for the microorganisms and mice, but must be fed previously to administration of the antagonist for maximum effectiveness. Briggs *et al.* (114) have compared the efficacy of crystalline natural CF, synthetic CF, and PGA for the chick and confirm the observations of Broquist *et al.* (113).

Occurrence.—Most of the available figures in the literature for the folic acid content of foods and other natural products have been obtained by bioassay with *S. faecalis* or *L. casei* or both and represent total folic acid (i.e., sum of activities of PGA, CF, etc.). An extensive compilation of such data has just appeared [Toepfer *et al.* (115)]. Since the discovery of CF[2], it has become important for some purposes to re-evaluate previous analyses and to consider new analyses in more specific terms. Wieland *et al.* (116) have described a differential microbiological method for FA and CF in which a correction for the CF activity of *S. faecalis* is used in order to obtain more specific values for FA. By analysis before and after incubation with an enzyme preparation, or after autolysis, an estimate as to the amounts of free and combined compounds present has been obtained. Dietrich *et al.* (117) also have published analyses for free and total FA and free and total CF on similar tissue preparations. It would appear that, while there are small amounts of free FA and perhaps also free CF in some tissues, both FA and CF are for the most part present in "bound form," likely as conjugates. Most tissues contain enzymes that upon incubation liberate the free compounds. In general, liver contains larger amounts of both FA and CF and their conjugates than do other tissues. There is some lack of agreement as to the approximate levels and ratios of FA to CF in tissues that will undoubtedly become clarified with additional work. Although there is chromatographic evidence for the presence in tissues of compounds other than PGA and CF capable of stimulating the organisms used for the assay, the principal activity results from compounds identical with synthetic CF or PGA. Dietrich *et al.* (118) have demonstrated that chick liver slices synthesize *L. citrovorum* activity at the same rate from pteroylglutamic acid, pteroyldiglutamic acid, and pteroyltriglutamic acid. Bioautographic analyses of the resulting total *L. citrovorum* activity yielded two growth zones in all cases. The fastest moving spot and leucovorin had the same R_F value; the third spot has not been identified. Apparently, all three substances tested have a common metabolic pathway to *L. citrovorum* activity, and more than one compound possessing CF activity is synthesized by chick liver. Since the "bound forms" of CF (conjugated CF) in yeast and liver preparations were found to move at the same rate when chromatographed by an ethanol-butanol-ammonia water developing agent, Doctor *et al.* (119) conclude that such preparations contain conjugated forms of CF that are identical.

Nichol (120) has been successful in preparing homogenates and active extracts from acetone powders of rat and chick livers which will anaerobically

convert PGA to CF as successfully as will formerly used liver slices [Welch & Nichol (1)]. The presence of ascorbic acid improves the yield. This system is being used to study the donors of the hydrogen and formyl involved in the biological conversion of PGA to CF, using chromatographic and counter current techniques. Rauen *et al.* (121) have identified N^{12}-formyl-folic acid as a product of incubation of PGA with pig liver homogenates. Jacobson & Good (122) have presented evidence that the incubation of PGA with a xanthine oxidase preparation from cream leads to the formation of a substance that is hematopoietically more active in pernicious anemia than is PGA. In the presence of hypoxanthine and methylene blue, the xanthine oxidase preparation is capable of reducing PGA, perhaps to its di- or tetrahydro form. Swendseid *et al.* (123) report that when suspensions of leucocytes or myeloid elements of bone marrow are incubated with PGA, under conditions similar to those described for liver slices, a substance is formed that stimulates the growth of *L. citrovorum* but that can be distinguished from CF (folinic acid) by the shape of the assay curve and by the capacity to reverse the inhibitory effect of chloromycetin under conditions in which folinic acid is not effective. The name "hemofolin" has been given to this hypothetical substance.

Biochemistry.—Further evidence has appeared indicating that folic acid is concerned in the following biochemical systems: (*a*) production of "formate" from glycine and the union of "formate" with glycine to produce serine; (*b*) methylation of ethanolamine to form choline, of homocysteine to form methionine, of nicotinamide to yield N^1-methylnicotinamide; (*c*) methylation of the pyrimidine ring to form thymine; (*d*) introduction of the two- and eight-carbon atoms into the purine ring; (*e*) introduction of the amidine carbon atom into histidine. The mechanisms involved are still not clear. It is clear, however, that the reactions involve one-carbon intermediates and FA[2] or its derivatives. Vitamin B_{12} is also implicated in some of these reactions. It has been speculated, but not proven, that FA participates in the transfer of one-carbon groups by reduction and reversible formylation.

Evidences of choline deficiency in the rat (hemorrhagic kidneys, fatty liver and high blood nonprotein nitrogen) have been prevented by addition of PGA plus vitamin B_{12} to the low choline-low protein diet [Fischer & Hall (124)]. Engel & Alexander (125) report that choline or 30 μg. of vitamin B_{12} plus 2 mg. of PGA per kg. of diet will prevent the nutritional edema and severe anemia occurring in rats maintained on diets low in choline and protein. PGA alone was inadequate. Stekol *et al.* (90) have studied the utilization of glycine-2-C^{14} and serine-3-C^{14} for the synthesis of choline, using FA and vitamin B_{12} deficient rats. They conclude that vitamin B_{12} deficiency reduces the utilization of the α-carbon of glycine but not the β-carbon of serine and that folic acid deficiency reduces the utilization of the β-carbon of serine and, to a smaller extent, the utilization of the α-carbon atom of glycine for choline formation. These results implicate vitamin B_{12} in the synthesis of serine from glycine and FA or its derivatives in the synthesis of

both the ethanolamine and methyl moieties of choline from serine and from glycine through serine. CF was used in these studies. Daft (126) has shown that mixtures of methionine, tryptophan, and threonine with adenine, or adenosine or yeast adenylic acid will lead to improvement in some symptoms of FA deficiency in the rat. The interpretation that FA is required for synthesis of these substances seems most likely. Broquist (127) has observed that the inhibition of growth of yeast by the folic acid antagonist aminopterin is overcome in a competitive fashion by CF and in a noncompetitive fashion by the step-wise addition of methionine, methionine plus purine bases and by methionine, purine bases, and histidine. These findings are interpreted to mean that aminopterin interferes with the reactions dependent upon CF and that this interference can be overcome either by adding CF or by adding the products formed through the action of CF, i.e., methionine, purine bases, and histidine, which are required by the organism for growth.

That the tissues of FA deficient rats and mice show a decreased capacity to methylate nicotinamide and to synthesize creatine has been observed by several investigators. Dietrich *et al.* (128) have found that with nicotinamide injections above 70 mg./kg. the excretion of N^1-methylnicotinamide by FA deficient rats is distinctly less than it is for normal animals. A 10 μg. injection of PGA returned the excretion to a normal level immediately (first 24-hour collection period) while 50 μg. of synthetic CF required six days to give the same response. Vitamin B_{12} was also effective but required eight to ten days. Fatterpaker *et al.* (129) report that FA deficient Swiss mice receiving nicotinamide excrete lower than normal amounts of the methylated derivative and that this amount can be increased by giving "formate" or methanol. PGA plus methanol gave the best response. It was also shown by use of liver homogenates that the capacity to synthesize N^1-methylnicotinamide was restored in the animals receiving supplements of methanol, "formate," or PGA. Similar experiments indicated that the *in vitro* capacity to synthesize creatine is also decreased in the livers of FA deficient mice and that the decrease can be prevented by PGA supplements or helped somewhat by "formate" or methanol injections [Fatterpaker *et al.* (130)].

Skipper *et al.* (131) have reported that PGA administered to mice during the two days previous to injection of C^{14}-formate and methopterin (a folic acid antagonist) partially reverses the inhibition of methopterin on nucleic acid synthesis, as indicated by C^{14}-formate incorporation. Lowe *et al.* (132), by the use of P^{32}, have presented data supporting the thesis that at least a part of the altered biochemistry of megaloblastic monkeys is a decreased capacity of liver to synthesize pentose nucleic acids. This capacity was restored to normal by PGA. Totter (133) has found that C^{14}-"formate" incorporation into *S. faecalis* cells grown on folic acid free media occurred in the presence of PGA or dehydroisoandrosterone but not in the presence of thymine. Increased PGA concentrations did not affect the uptake of C^{14} from "formate" unless suboptimum amounts of the vitamin were present.

This experiment is interpreted to mean that FA or dehydroisoandrosterone functions in the organism in the synthesis of thymine from "formate." Woods (134) has reviewed additional knowledge of the function of PGA and CF in one-carbon metabolism. Verly *et al.* (135) have studied the effect of PGA and CF on the synthesis of the labile methyl group.

Folic acid antagonists.—Folic acid antagonists are compounds closely related in chemical structure to FA[2] that interfere with some or all of the metabolic functions of this vitamin and its derivatives, i.e., PGA and CF. A large number of such compounds have been synthesized and are used extensively in producing experimental deficiencies in a wide variety of organisms, ranging from microorganisms to man, and as a means of studying the biochemical functions of PGA and CF. They have been used also in studies of the chemical pathology of pernicious anemia and other anemias and in the treatment and study of leukemia. An excellent summary of the chemistry of folic acid compounds and folic acid antagonists, general aspects of toxicity, reversibility of antagonism, hematologic effect, hormonal interaction, relation to enzyme action, mechanism of action, and effects on neoplasm has recently appeared and should be read by those interested in this field [Petering (136)]. Additional clinical reports on the therapeutic trials of folic acid antagonists continue to appear. A representative summary of this aspect of the subject is the *Proceedings of the Second Conference on Folic Acid Antagonists in the Treatment of Leukemia* (137). The relationships between vitamin B_{12}, FA, and CF have been reviewed by Girdwood (50, 138). A representative list of FA analogues, with their FA activity or antimetabolic activity, is to be found in the above review (136). 4-Aminopteroylglutamic acid is the most potent inhibitor of FA known at present and the most widely used for experimental purposes. Modification of the 4-amino-PGA structure has in every case decreased the inhibitory activity of the compound. However, in some cases, compounds more desirable for the chemotherapy of leukemia have resulted. Folic acid antagonists produce toxic symptoms in addition to those of FA deficiency, and these vary to some degree with species and sex. Quantitative studies of toxicity and the reversal of these effects with PGA and CF have been among the useful techniques for investigating the mode of action of PGA and its derivatives. Goldin *et al.* (139) have recently published a very thorough study of this type, using mice, the metabolites PGA and CF, and the antagonist aminopterin. They confirm previous studies (1) that indicated that aminopterin interferes in a noncompetitive manner with the conversion of PGA to CF. This is shown by the fact that, although PGA affords some protection when given one hour prior to aminopterin, it is ineffective when simultaneously administered; while CF, under such conditions, gives protection. Other data are presented to support this conclusion. It is also evident from these and other studies that aminopterin interferes with the use of CF. While this action is predominantly competitive in type, there is evidence in this case, too, that aminopterin slowly interferes in a noncompetitive manner. Swendseid *et al.*

(140) have shown that the excretion of aminopterin continues for several days after discontinuance of the dose and that FA excretion is greatly increased by the administration of the drug to leukemic patients. This supports the general conclusion from previous work, that the toxicity of aminopterin is primarily a result of replacement of FA or derivatives, i.e., CF in the structure of one or more enzymes, coenzymes, or essential metabolic intermediates. Wright (141) has observed a reduction of eosinophiles in many patients receiving the antagonist Amethopterin and, therefore, has suggested that some of the effects of these compounds may be mediated through the adrenal cortex. The treatment of acute leukemias of children with folic acid antagonists continues to be one of the most useful methods of obtaining remissions in this disease, although the response to this treatment is not uniformly successful; and although it does not cure the disease, it does help in the care of such patients. Most importantly of all perhaps, these studies have stimulated investigations and pointed up the possibilities of the chemotherapeutic approach to the problem of malignant disease. Such antagonists have not been found useful in the treatment of solid tumors [Schoenbach *et al.* (142)].

Hamilton *et al.* (143), Bieber *et al.* (144), and Hitchings *et al.* (145) have shown that the 2,4-diaminopyrimidines, which have been found to be competitive antagonists of FA for *L. casei*, also produce hematological changes in the rat and the dog as does the folic acid antagonist (aminopterin). Such compounds also inhibit the development of frog embryos at certain stages. This is interpreted as an inhibition of the biosynthesis of nucleic acids. Toxicity and reversal studies with *S. faecalis* have led to the hypothesis that the relationship between these pyrimidines and CF may be analogous to that between aminopterin and PGA, i.e., that the metabolite with which they compete may be more complex than either PGA or CF. Morgan (146) has reported that the inhibitory action of sulfadiazine on the growth of psittacosis virus (6Bc) in embryonated eggs is readily reversed by CF but not by small amounts of B_{12}. It had been shown previously that PGA and *p*-aminobenzoic acid were also effective in this respect, the latter compound on a competitive basis. A number of folic acid antagonists, in concentrations nontoxic for the host tissue, were found to inhibit the growth of this virus in tissue culture. CF readily overcame this inhibition. The advantages of the tissue culture technique in investigations of this type are discussed. Similar experiments indicated that various amounts and mixtures of thymine, thymidine, adenine, guanine, xanthine, hypoxanthine, cytidylic acid, and digests of desoxyribonucleic acid were ineffective in influencing the inhibition of the growth of the virus by sulfadiazine [Morgan (147)]. It will be recalled that these compounds do reverse the inhibitory action of sulfa drugs on some microorganisms. A number of purine analogues inhibited the growth of the psittacosis virus at concentrations which produced no obvious toxic effects on the tissue culture. Some of these effects were reversed by adenine or guanine or both. Lascelles & Woods (148) have produced more

evidence to indicate that although the details of the mechanism by which sulfa compounds inhibit growth in bacteria are complex and vary with species, they are intimately connected with FA synthesis and use. Shive (149) and Woods (134) have recently reviewed this subject in detail.

Miscellaneous.—The interrelationships of factors that influence the megaloblastic anemias have been reviewed by Girdwood (50). Thompson (150) has demonstrated that PGA has a direct maturing effect on cultures of megaloblasts obtained from patients suffering from pernicious anemia in relapse. No effect was observed with either liver extracts, vitamin B_{12}, or thymine, all preparations which at least under some circumstances are effective in megaloblastic to normoblastic erythropoiesis *in vivo*. This is interpreted as indicating that these latter materials function indirectly. It is known that vitamin B_{12} must be bound to intrinsic factor or an analogous factor before it is effective in this respect. Folic acid, in addition to vitamin B_{12}, has been found necessary for maximum hematopoietic effect in mice made anemic by phenylhydrazine [Vijayaraghavan & Dunn (96)]. Sundberg *et al.* (151) have described in considerable detail the previously reported production and repair of experimental megaloblastic anemia in monkeys that resembles in many respects the secondary megaloblastic anemias of infancy, pregnancy, sprue, and gastrectomy. The anemia was produced by deprivation of adequate amounts of ascorbic acid along with feeding of a milk diet known to contain only small amounts of folic acid. Vitamin B_{12} was not effective therapeutically nor prophylactically. PGA or CF caused a prompt reversion of the megaloblastic marrow to a normoblastic marrow. Ascorbic acid also gave such a response but more slowly. CF[2] proved to be an effective therapeutic agent in doses smaller than those of PGA[2]. Although PGA returned the marrow and blood to normal, it did not alleviate lesions of scurvy or other symptoms. The megaloblastic anemia was produced in some animals receiving adequate ascorbic acid. An anemia occurred in scorbutic animals receiving PGA, but it was not of a megaloblastic type. It seems evident, as previously pointed out (1), that a deficiency of FA or its derivative CF is the primary cause of the anemia and that ascorbic acid is somehow concerned with the efficient use of this vitamin. Therefore, in the absence of ascorbic acid, a given level of folic acid is less adequate in protecting the animal.

Spray & Witts (152) have measured the FA content in plasma and in urine after doses of 1 mg. of PGA administered to 12 normal people, 9 untreated, and 9 treated patients with pernicious anemia, and 5 patients with steatorrhea. The normal and treated patients showed a large increase in plasma level that returned to normal in five hours, and 2 to 20 percent excretion of FA in the urine in five hours. The untreated cases and patients with steatorrhea showed slight elevation in the blood and very little excretion. This could be explained either as being because of impaired absorption or increased utilization of PGA in pernicious anemia in relapse. It is presumed that the low levels in steatorrhea resulted from faulty absorption.

According to Girdwood (153), CF administered orally to normal subjects or to sprue patients with free hydrochloric acid in the stomach, is excreted largely as FA. In pernicious anemia patients and in patients without pernicious anemia but with histamine-fast achlorhydria, it appears in serum and urine primarily as CF. Injected CF appeared in serum and urine as CF. It has been shown that the orally administered, naturally occurring conjugate, pteroylhexaglutamyl-glutamic acid, is excreted as FA at about the same level as when an equivalent amount of PGA is given. However, Spray (154) reports that the same FA conjugate in yeast extract and presumably in other natural foods and preparations is apparently not as readily absorbed since the administration of similar doses derived from these sources resulted in lower excretion values.

The excretion of FA following test doses of the vitamin is significantly lower in both acute and chronic leukemia than in normal subjects, thus suggesting an increased demand under these conditions [Swendseid *et al.* (155)]. This is consistent with previous observations that the CF content of leukemic cells is higher than that of normal cells of the same type (1).

6-Formylpteridine, the primary photogenic product of ultraviolet irradiation of PGA, is a remarkably effective inhibitor of xanthine oxidase *in vitro* (1). Since many preparations of PGA contain significant amounts of this compound, it was suggested that the apparently decreased xanthine oxidase content of livers of animals receiving high levels of FA might be a result of this effect. Dietrich *et al.* (156) have shown that this can not be the case.

Folic acid deficiency has been produced in the lamb by use of sulfathalidine. The symptoms are those described for other animals [Draper & Johnson (157)]. Drosophila requires FA and can use PGA, the triglutamate, or CF [Hinton (158)]. Folic acid deficient rats produce less complement fixing murine typhus antibodies than do paired weight controls [Wertman *et al.* (159)].

FA deficiency in the rat, induced by succinylsulfathiazole, produces a marked increase in the urinary excretion of a compound of as yet undetermined structure which, upon heating, yields glutamic acid. The compound has been partially purified as the barium salt, appears to contain two moles of glutamic acid and two amide groups, and has a molecular weight of about 500 [Silverman *et al.* (160)]. Clegg *et al.* (161) claim to have developed an improved medium for FA assay by L. *casei*. According to Nelson *et al.* (162), a high percentage of congenital abnormalities appeared in the young of rats made deficient in FA between the ninth and eleventh day after breeding. Earlier deficiency led to fetal death; deficiency after the twelfth day resulted in normal litters.

Ascorbic Acid

Chemistry.—Numerous modifications of existing methods and innovations for the determination of ascorbic acid have appeared. Few of them seem to possess any real advantages over established procedures. The method of Roe

et al. (163), which had been adapted to microtechniques by Bessey *et al.* (164), has been further modified, eliminating the need for specially prepared carbon black [Lowry *et al.* (165); Meyer *et al.* (166)]. The commonly employed histological demonstration of ascorbic acid depends upon the reduction of acid silver nitrate. Conclusions drawn from the deposition of silver granules, using this stain, have been questioned previously. Reiner (167) has re-examined the acid silver nitrate method with particular reference to specificity, sensitivity, and localizability. It may be concluded from his findings that a fair, although far from perfect, degree of specificity exists but that diffusion phenomena are such that no deductions, based on localized precipitates of silver in tissue sections, can be safely made. This finding thus sheds added doubt on the question of localization of ascorbic acid in specific regions of the cell since this may merely represent a surface phenomenon.

Good yields of dehydro-L-ascorbic acid (DHA) have been obtained by removal of the solvent of crystallization from the crystalline "methanol complex" of DHA,[2] which, in turn, is prepared by chlorine oxidation of ascorbic acid [Pecherer (168)]. Deuterated ascorbic acid has been prepared and its infrared spectrum studied [Weigl (169)]. One of the hydrogen atoms directly bonded to carbon was found to be quite labile and readily exchangeable. This should be of interest in any future investigative application of ascorbic acid labeled with isotopic hydrogen and may be of more direct biochemical significance. Ascorbic acid has been prepared also with a C^{14} label in position one [Burns & King (170); Salomon *et al.* (171)] and with a uniform enrichment with C^{14} in all positions [Bothner-By *et al.* (172)].

Tyrosine metabolism.—As in previous years, attempts have been made to link ascorbic acid to some specific function in enzyme systems. The reported interaction of ascorbic acid with urease [Rao & Giri (173)] and with choline oxidase [Williams (174, 175)] remains speculative. Similarly, a specific function for ascorbic acid in the inhibition of xanthine oxidase can not be considered as established [Feigelson (176)].

It has been known for a number of years that ascorbic acid can favorably affect the tyrosyluria of vitamin C deficient infants and guinea pigs. In addition, more recent work seems to indicate strongly the participation of ascorbic acid in tryosine metabolism. Also, similar results have been obtained *in vitro* with liver preparations. Hird & Rowsell (177), LeMay-Knox & Knox (178, 179), La Du *et al.* (180, 181), and Schepartz (182) agree that the first step of tyrosine catabolism involves transamination. They, as well as Sealock *et al.* (183), Painter & Zilva (184), and Rienits (185) show that ascorbic acid catalyzes the subsequent oxidative steps. The oxidative capacity of liver preparations has been studied in some detail by Sealock *et al.* (183), based on the work of Sumerwell & Sealock (186) who have found appreciable amounts of ascorbic acid bound to proteins. Oxygen uptake by liver homogenates from normal and scorbutic guinea pigs, and dialyzed and undialyzed liver extracts prepared from normal liver acetone powder, were measured.

The scorbutic homogenates were found to oxidize tyrosine almost as completely as did those from normal animals, presumably as a result of the presence of bound ascorbic acid (approximately one-third of the total ascorbic acid present, or one half of the quantity of bound vitamin in normal homogenates). Dialysis diminished both free and bound ascorbic acid considerably and depressed the initial rate of oxidation. Addition of ascorbic acid to dialyzed or undialyzed extracts increased oxygen uptake, especially in the former. It was concluded that the relatively high degree of tyrosine oxidation by these preparations was attributable to the presence of sufficient quantities of the bound vitamin. In view of similar effects of D-isoascorbic acid, D-glucoascorbic acid, and reductone, a coenzyme function of ascorbic acid in tyrosine catabolism was proposed, with the enediol structure as a specific requirement. Work of Udenfriend *et al.* (187) has shown that the oxidation of aromatic compounds can be carried out with oxygen in a "model system" containing ascorbic acid and is catalyzed by ferrous (II), ferric (III), cupric (II), or cobaltous (II) ions. In view of the accelerating effect of hydrogen peroxide on the initial reaction rate, a peroxide intermediate was postulated. *In vivo*, these aromatic compounds, which are metabolized largely through hydroxylation, were found to disappear at a slower rate from the plasma of scorbutic guinea pigs than from the plasma of normal guinea pigs. Ascorbic acid, but not isoascorbic acid, restored the oxidation to normal. Similarly, in liver slices of scorbutic guinea pigs, impaired oxidation was found [Axelrod *et al.* (188)]. Thus, it appears that the effect of ascorbic acid is less precisely defined than might be indicated by the work of Sealock *et al.* (183). Additional evidence for the complexity of the problem arises from the findings of Suda *et al.* (189), who have shown that ferrous (II) iron in liver preparations from scorbutic guinea pigs is more effective than is ascorbic acid in restoring the depressed homogentisicase activity to normal. Other substances, e.g., folic acid [Woodruff *et al.* (190)], vitamin B_{12} [Govan & Gordon (191)], ACTH (adrenocorticotropic hormone) [Levine *et al.* (192)], are effective in eliminating tyrosyluria, although less efficiently than is ascorbic acid. D-Isoascorbic acid (178), D-glucoascorbic acid, and reductone (183) and hydroquinone (181) have been shown to be capable of replacing ascorbic acid *in vitro* but not *in vivo*. Such low structural requirements on the part of an enzyme are surprising indeed. Lastly, the nature of the ascorbic acid-protein complex of Sumerwell & Sealock (186) has not been sufficiently investigated, particularly with the possibility of artifacts in mind. It would appear that present information does not permit unqualified conclusions as to the nature of interaction of ascorbic acid with the enzyme systems metabolizing tyrosine.

Folic acid metabolism.—The close, but unexplained relationship of ascorbic acid to the folic acid group of vitamins mentioned above is by no means the only observation linking the activities of these two vitamins. Hemorrhagic tendencies of folic acid deficient rats have been beneficially influenced and normal white cell and normoblast counts restored by administration of ascorbic acid. In a series of papers by Williams (193) and Schwartz & Wil-

liams (194, 195, 196), the view has been taken that folic acid deficiency inhibits the biosynthesis of ascorbic acid. Aminopterin fed to rats resulted in a lowering of liver ascorbic acid levels to approximately one half those found in normal livers. Urinary ascorbic acid excretion values fell off proportionately as the liver concentration decreased. Sulfasuxidine lowered liver ascorbic acid levels in much the same way as did aminopterin. Based on the low solubility of sulfasuxidine, it was concluded that the effect on liver ascorbic acid may be attributed to intestinal bacteriostasis, resulting in inhibition of production of one or more substances needed by the rat for the synthesis of ascorbic acid. Whether the lowered vitamin levels in the livers of drug fed rats actually signify a direct involvement of folic acid or related compounds remains problematical. Other substances, in particular hepatotonic materials, are known to affect liver ascorbic acid levels similarly [Sapeika (197)] while 1,2,5,6-dibenzanthracene, a carcinogenic hydrocarbon, has been reported to cause significant increases [Elson *et al.* (198)] or no change [Meduski (199)].

Rather than the members of the folic acid group functioning in ascorbic acid synthesis, the work of May *et al.* (200, 201), Sundberg *et al.* (151), and Proehl & May (202) indicates the converse. The folic acid and iron requirements of monkeys were increased, and it was found possible to produce megaloblastic anemia by feeding diets deficient in vitamin C and low in folic acid, although the total folic acid content was adequate when ascorbic acid supplements were given. Neither vitamin antagonists nor drugs were employed. Either PGA (pteroylglutamic acid) or ascorbic acid caused a reversion of the megaloblastic marrow to normal, PGA acting more promptly without otherwise altering the course of scurvy. Folinic acid (citrovorum factor, CF) was active in smaller doses than was PGA, but vitamin B_{12} alone proved ineffective prophylactically and therapeutically. Conversely, scurvy and anemia without megaloblastic marrow developed when folic acid intake was adequate but vitamin C was deficient. The authors suggest, on this basis, that a chronic vitamin C deficiency leads to a deficiency of folic acid or a disturbance in the metabolism of folic acid or related compounds, and thus results in megaloblastosis. Consistent with this, Nichol & Welch (203) have shown that liver preparations from either normal or folic acid deficient rats are capable of producing a factor essential for *Leuconostoc citrovorum* 8081 (CF)[2] and that considerably more is synthesized on incubation with PGA together with ascorbic acid than with either vitamin alone. In the treatment of 36 cases of infantile scurvy with megaloblastic marrows, Zuelzer *et al.* (204) have concluded that ascorbic acid does not have an hematopoietic function, and its deficiency does not, *per se*, lead to anemia, since PGA is as effective as PGA plus ascorbic acid, but ascorbic acid without PGA gives no improvement. These results are not necessarily in conflict with the conclusions of May and co-workers (151, 200, 201, 202) since the unstated therapeutically effective doses of PGA employed may have been larger than those which would suffice with adequate vitamin C intake. The *in vivo* conversion of PGA to CF, entirely analogous to that observed *in vitro*, has been studied by Bro-

quist *et al.* (205) and Welch *et al.* (206). The former found, by microbiological assay of the urine of six normal men, that large amounts of PGA ingested orally led to a sharp increase of CF over normally observed values. On the other hand, administration of PGA and ascorbic acid combined increased the excretion values two to three times over those obtained with PGA alone. [However, ingestion of leucovorin (synthetic CF) alone gave higher excretion values of CF and total folic acid than did leucovorin with ascorbic acid.] Welch *et al.* (206) concur in the finding of augmented urinary CF of rats and human subjects when ascorbic acid and PGA are administered simultaneously. In contrast, two adult scorbutic patients failed to respond to PGA and ascorbic acid administration with highly increased CF excretion; PGA alone also led to only small amounts of CF in the urine. It is of interest that in the rat, glucoascorbic acid, but not glutathione or cysteine, was equally as effective as ascorbic acid in increasing CF excretion.

Recently, Hill & Scott (207) have reported the existence of CF-liberating enzymes in hog kidney and chicken liver, of which only the latter appears to be activated by ascorbic acid. The bulk of these studies present a consistent picture. However, more work will be required to establish definitely the role of ascorbic acid in the transformation of folic acid.

Collagen metabolism.—In view of the efficacy of ACTH[2] and cortisone in rheumatoid arthritis [Hench *et al.* (208)], it was to be expected that tests should be performed on the development and repair of scorbutic lesions in guinea pigs when treated with these substances rather than with ascorbic acid. Some aspects of this problem will be mentioned in a later section; the discussion here will be confined to effects on lesions in scurvy that resemble those of arthritis, the healing of wounds of scorbutic guinea pigs, and formalin arthritis.

Schaffenburg *et al.* (209) have studied the development of lesions in four groups of guinea pigs, three groups of which were on a diet poor in vitamin C to produce a state of "chronic scurvy," one group being normal control animals. Two groups of animals on the vitamin-poor diet also received DCA (desoxycorticosterone acetate) or cortisone acetate. It was found that of the animals on unsupplemented scorbutogenic diet, only those receiving DCA or no other treatment presented symptoms of scurvy, these being more severe and appearing earlier in the DCA group. Cortisone-treated animals did not differ, in general, from normal control animals. Cortisone, therefore, was found to possess considerable antiscorbutic properties, judged by body weight changes, joint swelling, hemorrhages, and weight of adrenal glands. DCA, on the other hand, proved to be conducive to the development of scorbutic lesions. It was concluded that the symptoms of scurvy are apparently secondary to those of adrenal insufficiency and result from hormonal imbalance, i.e., continued production of DOC (desoxycorticosterone) without adequate formation of carbohydrate-active steroids. A similar study has been carried out by Herrick *et al.* (210) with guinea pigs fed a scorbutogenic diet, either unsupplemented, or supplemented with ascorbic acid or with cortisone, after

five days on the vitamin C free regimen. All scorbutic control guinea pigs died within 12 to 19 days while those receiving cortisone survived considerably longer (37 to 50 days). No abnormalities were found in the latter group except in the adrenal glands, which presented a picture of atrophied cells, shrunken nuclei, and decrease in the number of cytoplasmic granules and lipid vacuoles. The unusually short survival period of the untreated animals raises a doubt as to the existence of a pure vitamin C deficiency state, and makes it likely that a complicating factor co-existed. Similar results were obtained previously by Hyman *et al.* (211), who studied both the effect of cortisone and ACTH. In this case, adrenal glands of ACTH-treated animals were hypertrophied to a somewhat greater extent than those of untreated scorbutic animals. Eisenstein & Shank (212) have found the onset of severe scurvy and mean survival period to be increased by ACTH and have reasoned that this implies the existence of a functioning adrenal cortex despite vitamin C deficiency. Recently, Hughes *et al.* (213) have studied the results of DCA, ACTH, and cortisone acetate on the development of "arthritic" lesions in female guinea pigs on vitamin C deficient diet. The protective power of cortisone was found to be greater than that of ACTH while DOC exacerbated the condition, in the doses used. The conclusion was reached that a catalytic role of ascrobic acid in the production of "oxy-type" adrenocortical hormones exists. Contrary to these findings, Clayton & Prunty (214) have obtained no considerable effect of ACTH or cortisone on weight loss, survival rate, or symptoms of scurvy in male or female guinea pigs on a scorbutogenic diet. Their conclusions are, nevertheless, the same as those of Eisenstein & Shank (212) because of their observation of the increasing 17-ketosteroid excretion during the progress of scurvy. Essentially similar results have been shown in the researches of others. Nigeon-Dureuil *et al.* (215) have found no effect of ACTH on weight loss, appearance of symptoms of scurvy, or time of death. Hemorrhagic lesions, if anything, were more pronounced in animals receiving ACTH. Wolbach & Maddock (216) have found that for guinea pigs on a scorbutogenic diet, neither daily prophylactic doses of 2.5 to 10 mg. of cortisone per kg. of body weight nor 60 to 140 mg. per kg. after 18 to 22 days, influence the symptoms of scurvy, as compared with those of untreated control animals. Ascorbic acid administered alone was as efficient in causing repair as was ascorbic acid together with cortisone. The healing of nonperforating wounds in the cornea has been found to be delayed in control as compared with scorbutic guinea pigs, both treated with cortisone, but no material differences have been noted, in general, by Barber & Nothacker (217).

Upton & Coon (218) placed guinea pigs on a scorbutogenic diet with and without vitamin C supplements. An experimental wound was inflicted on the twelfth day of feeding the deficient diet and ACTH or cortisone administration was started. No effect of ACTH or cortisone on the healing of wounds in normal or scorbutic animals was discerned; wounds of the latter had little collagen and failed to heal. The authors suggest that this casts doubt on the antiscorbutic properties of cortisone, which may represent only protection against the nonspecific stress of vitamin C depletion.

This spectrum of results is probably attributable, in part, to differences in criteria used to evaluate the effect of cortisone, or ACTH (which presumably sparks the elaboration of endogenous cortisone). Partly, also, the divergences may be a result of basic factors in the design of the experiments, e.g.,timing of hormone injections. However, a specific beneficial influence of these hormones in preventing or curing the collagen changes accompanying (or possibly constituting) scurvy, has not been established. Such changes as have been observed may well be due to relief of nonspecific symptoms of prolonged stress, rather than to any specific antiscorbutic properties of the hormones. It would be surprising, also, to find that cortisone or ACTH should stay or reverse the collagen damages of scorbutic guinea pigs when adverse results of these hormones on healing of wounds appear to be generally agreed upon. This has been shown for species susceptible to scurvy, as man [Ragan *et al.* (219)] and the guinea pig [Wolbach *et al.* (216)], as well as for the rabbit [Ragan *et al.* (220)], the mouse [Spain *et al.* (221)], and the rat [Baker & Whitaker (222)].

Following the publication by Selye (223) of his studies on the production of arthritis induced by formalin injection, a number of workers carried out experiments to test the effect of ascorbic acid, singly or combined with steroid hormones, on induced arthritis. Cortisone and ACTH had been shown, by Selye, to prevent the acute and the chronic stages while DCA exacerbated the condition. However, Parkes & Wrigley (224) disagree on certain observations regarding the course of formalin arthritis, and on the effect of ACTH and cortisone, which were found not to produce tangible improvement. DCA, alone or with ascorbic acid, was found to result in aggravation. Dugal (225) has found that exposure to cold considerably aggravates formalin arthritis in the rat and that treatment with ascorbic acid greatly diminishes the consequences of formalin injection in adult, but not in young animals. A "cortisone-stimulating effect" of ascorbic acid was suggested, admittedly on a purely speculative basis. Siebenmann (226) finds that both cortisone and ascorbic acid, but not DOC[2] with or without ascorbic acid, are of benefit in the acute and chronic stages. In contrast to the hypothesis advanced by Dugal (225), cortisone administration gave results differing markedly from those of ascorbic acid, the latter leading to maturation of granulation tissue and deposition of collagen fibrils. In addition, the effect of ascorbic acid was not dependent on the presence of the adrenal glands, i.e., could not have resulted from any adrenocortical stimulation nor prevention of adrenocortical hormone destruction. In this connection, the findings of Brownlee (227) are pertinent; he has concluded that ascorbic acid plus DCA gives protection to both normal and adrenalectomized rats, ascorbic acid gives some slight protection to normal rats only, and DCA exacerbates the formalin-induced arthritis in normal rats, but only slightly in adrenalectomized animals. However, Coutu & Selye (228) have failed to confirm the statistical significance of these observations. A protective action of DOC and ascorbic acid, administered simultaneously, has been claimed also by Gross (229) while Anttonen & Rinne (230) and others do not concur. The data of

Bacchus (231) are apparently in essential agreement with those of Siebenmann (226) as regards the effects of cortisone and ascorbic acid. Bacchus (231), additionally, has investigated the effect of "potassium chloride-flooding" and finds that of the three treatments, cortisone administration following formalin injection results in much less swelling and inflammatory changes in the periarticular tissues than when the other methods are employed.

The involvement of ascorbic acid in the deposition and maintenance of collagen has also been studied, unencumbered by other complicating conditions. Not the least strongly reflected is our uncertain state of knowledge of the functions of ascorbic acid in its relation to collagen, its most clearly obvious and one of its most vital roles. In an examination of the collagen contents of various organs of scorbutic guinea pigs, as compared with normal control animals, Elster (232) has found that lungs and livers of the scorbutic animals have markedly lowered organ weight and collagen content as compared with those of age controls, but equal to those of weight controls. Spleens did not differ, either as to weight or collagen content. Kidneys had weights approximating those of age controls, but a collagen content resembling that of weight controls, while heart weights were similar to those of weight controls, although their collagen content resembled that of the age controls. On a percentage basis, the collagen of skeletal muscle was increased over either control group. It was pointed out that in no organ was there a greater loss of collagen than of other tissue constituents. In fact, muscular tissue appeared to be more sensitive to destruction than collagen itself. Hence, it has been concluded that, although ascorbic acid unquestionably is essential in collagen formation, it may not be in collagen maintenance. Robertson (233) has studied guinea pigs suffering from chronic or acute scurvy and comes to essentially identical conclusions. Additionally, the decrease of collagen observed in teeth and costochondral junctions of deficient guinea pigs was ascribed to the continuation of growth, and hence, dilution of already existing collagen. The extent to which the method for collagen determination [Lowry *et al.* (234)], employed by him as well as by Elster, can detect more subtle changes was, however, left open to doubt, and this reservation must be borne in mind in the interpretation of these, as well as of the subsequent data of Robertson (235, 236). The stability of previously formed collagen during scurvy has been studied by Robertson (235) in the guinea pig. Collagen formation was stimulated by experimental wounds, by subcutaneous injection of diacetyl phosphate or Irish moss extract, and by wrapping the kidneys with plastic. Subsequent to healing, the collagen content of each site was compared in a group of animals made scorbutic with those continuing to receive ascorbic acid. No differences were found between the two groups except in the perirenal collagen, which was decreased in the scorbutic animals. This decrease was ascribed to stimulation continued during the scorbutic state when formation of collagen was impeded. Robertson (236) has found also that glycine-N^{15} is incorporated at a higher level into the collagen of normal guinea pigs than into the collagen of animals on a vitamin

C deficient diet or on a restricted caloric intake, but that there is no effect specifically attributable to ascorbic acid. This would appear to be in contradiction to a large body of previous results but the suggested explanation, an exchange in the peripheral fibers rather than a complete resynthesis of the macromolecules, skirts this difficulty.

That the use of more rigid criteria is called for in order to define what is meant by "collagen" is indicated by the work of Gersh & Catchpole (237) who have obtained evidence that depolymerization of connective tissue ground substance does, in fact, occur in scurvy. Furthermore, Pirani & Catchpole (238) have suggested that the increased levels of serum glycoproteins found in acutely or chronically scorbutic guinea pigs may be a result of depolymerization of the ground substance of normally formed glycoproteins, thus supporting Gersh & Catchpole (237), but concede that these effects may also be attributable to failure of the anabolic process, or both. More studies could be cited. It may suffice to reiterate the uncertainty of our information, based largely, it appears, on the uncertainty of the assay procedures. The formation of mucopolysaccharides has been found to be decreased in the scorbutic guinea pig [Penney & Balfour (239)], possibly of interest with regard to the findings of Gersh & Catchpole (237) and Pirani & Catchpole (238). Pirani *et al.* (240), investigating the nature of scorbutic arthropathy in the guinea pig, have found that immobilization of knee joints does not lessen the severity of the changes compared with those in free extremities. However, the cast was applied only after 10 days on the deficient diet. Elster & Schack (241) have studied capillary permeability of vitamin C deficient guinea pigs, using T-1824 (Evans blue), without arriving at any definite conclusion except to note that dye diffusion and blood volume are normal.

Adrenal function.—Some aspects of the relation of ACTH[2] and steroid hormones to ascorbic acid have been discussed in a previous section in regard to their bearing on the genesis, prevention, or cure of scorbutic lesions or conditions resembling them in the guinea pig and man. These, however, are only one facet of a rapidly expanding literature that seeks to explore the question of ascorbic acid-adrenocortical interaction. The field has been reviewed recently by Pirani (242). Elevated levels of urinary steroids, as well as certain alterations in the adrenal glands, e.g., hypertrophy, fall of ascorbic acid concentration and cholesterol concentration, changes in the blood picture, such as eosinopenia, or increased resistance to "stress" have been taken to indicate accelerated adrenocortical activity. Valuable information on the role of adrenal ascorbic acid might be gained from studies on the activity of the adrenals in scurvy, i.e., by settling the problem of hyperfunction versus hypofunction in this deficiency state.

Using the pair-feeding technique, Banerjee & Deb (243) have shown that both adrenal ascorbic acid and cholesterol are decreased in guinea pigs with presumably severe scurvy. This was taken as an indication of reduced adrenal activity, that is, the impaired carbohydrate metabolism of such

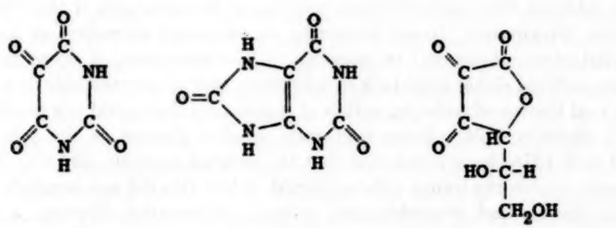
animals was suggested to be a result of a combined deficiency of insulin and cortical hormones. Consistent with this, in a later paper (244) these workers report significantly decreased urinary 17-ketosteroid levels in female scorbutic guinea pigs, compared with pair-fed control animals. The point is not too well taken, however, because reduced adrenal ascorbic acid and cholesterol have been generally taken as an indication of accelerated adrenal activity. Also, Long (245) and Oesterling & Long (246) have been able to show an essentially normal response of adrenal cholesterol to ACTH in the early stages of scurvy while the low levels of ascorbic acid appear to be "inert." The urinary excretion of adrenocortical products is probably best studied in female animals, as Banerjee *et al.* (244) have done. Nevertheless, Clayton & Prunty (214) have found increased urinary ketosteroids in deficient animals, female as well as male, rising to a peak in the terminal phases. ACTH injections resulted in increased 17-ketosteroid excretion levels. By these criteria, and hypertrophied glands, there was no evidence of reduced adrenocortical function as a result of ascorbic acid depletion. Formaldehydogenic corticoid excretion was found to be considerably elevated above the values of weight-paired animals, in studies by Nadel & Schneider (247) on male guinea pigs. Administration of ascorbic acid resulted in depressed excretion values, rising again on cessation of vitamin feeding. It was proposed that adrenocortical function does not require ascorbic acid under these conditions of severe "stress." Curiously, starved animals, even when moribund, did not show nearly so large an increase of formaldehydogenic substances in the urine. The suggestion was made that ascorbic acid deficiency constituted the chief "stress," presumably in addition to the inanition "stress," but leaves much unexplained. Bacchus *et al.* (248) believe the function of ascorbic acid to be related to the breakdown of cortical hormones, i.e., to result in diminished degradative changes in cortical hormones and thereby reduce 17-ketosteroid excretion. This important aspect, increased steroid excretion under various conditions, on which so many conclusions have been based, remains to be satisfactorily explained. It can not be doubted that the adrenal glands when subjected to the action of ACTH, i.e., under "stress," do elaborate increased amounts of cortical steroids. Urinary levels of steroids also increase under such circumstances, and the correlation is quite well established. Whether the converse is true, that unusually high urinary steroid levels inevitably connote a condition of adrenal hyperactivity, does not appear to be equally well founded. The extent to which such generalizations apply would seem to be open to further investigations, aimed at clarification of the influence of other factors on steroid excretion. There is evidence that the quantities excreted actually represent only a small portion of the adrenocortical steroids synthesized [Vogt (249)], thus leaving room for even relatively minor metabolic changes to produce marked differences in steroid excretion without affecting synthetic processes directly. Studying the state of activity of the adrenal cortex, as judged by 17-ketosteroid excretion (either spontaneous or with ACTH stimulation) in scorbutic monkeys, Stewart *et al.* (250)

have found values equal to or better than those of normal monkeys, in general. Based on this, as well as progressive eosinopenia in the peripheral blood, normal or increased function of the adrenal glands in vitamin C deficient monkeys was believed to exist. Reservations were made, however, with respect to the eosinophile decrease because of its uncertainty as an indicator of adrenocortical activity. In the normal human adult, Kayahan (251) has found that 4 gm. of ascorbic acid *per os*, daily for four days, increases glucocorticoid excretion while decreasing 17-ketosteroids. This result is interesting with respect to the conclusions of Bacchus *et al.* (248). Kayahan believes that, in view of the differences of ACTH action on the adrenals from that of ascorbic acid, the action of ascorbic acid must be directed at the adrenals rather than the pituitary. The increased survival period of scorbutic guinea pigs when treated with ACTH or steroid hormones, which has been observed by some workers and discussed before, also has been interpreted to signify unimpaired adrenal function even when the vitamin is lacking. Hence, it was concluded that ascorbic acid was nonessential for the functions of the gland. Conversely, increased excretion of ascorbic acid has been noted in rats exposed to low temperatures by Thérien & Dugal (252); Monier & Weiss (253) have noted an increase in urinary ascorbic acid, DHA,[2] and DKA (diketo-L-gulonic acid) under similar conditions; and Beck *et al.* (254) have reported that humans treated with ACTH also excrete increased amounts of ascorbic acid. On the basis of excretion of total ascorbic acid and decomposition products, Salomon (255) has found no significant changes in guinea pigs or hypophysectomized rats, following administration of ACTH, nor in guinea pigs suffering from the consequences of diphtheria toxin administration. The efficacy of ascorbic acid under a variety of stressful conditions, including burns and injection with bacterial toxins, has been known for some time, although some controversy exists on the topic. More recently, Dugal & Thérien (256) have shown that injection of 150 mg. of ascorbic acid per day can prevent adrenal hypertrophy, one of the manifestations of the adaptation syndrome, in rats exposed to low temperatures (−1°C.) for a prolonged period (40 days), while increasing cold resistance at the same time, judged by the survival of 75 per cent of the treated as against none of the untreated rats. Similar results were obtained with animals exposed to 4°C. for 240 days, and with guinea pigs receiving 25 mg. of ascorbic acid per day. In monkeys, large doses of ascorbic acid also gave protection against exposure to cold, although pre-exposure was of no effect [Dugal & Fortier (257)]. Dugal & Thérien (258) also have shown that in normal rats the injection of 150 mg. of sodium ascorbate per day does not affect the adrenal weight-increasing activity of 10 mg. of ACTH per day, over a three day period, i.e., does not prevent the action of ACTH. However, in hypophysectomized rats, such a dose of sodium ascorbate potentiated the action of 0.5 mg. of ACTH, judged by significantly increased adrenal weights. This small dose of ACTH, ineffective alone or in combination with sodium bicarbonate, was "activated" by ascorbic acid. The apparent contradiction to other results (256,

257) remains to be clarified. Histological studies on the adrenals of these animals (258) have been reported by Des Marais & Leblanc (259), who also have found *in vitro* evidence supporting the potentiating effect of ascorbic acid on ACTH. In differently designed experiments, Eisenstein & Shank (212) have found that the adrenal weight bears an inversely proportional relationship to ascorbic acid intake in ACTH treated guinea pigs. Since the number of circulating eosinophiles progressively decreased with advancing severity of the vitamin C deficiency state and also resulted in delay of onset of scurvy and increased mean survival period, these authors concluded that ascorbic acid is not directly essential to the elaboration of C-11 oxygenated steroids, and that scurvy represents a nonspecific stress. Eisenstein & Boniface (260), using rats, also have shown the prevention of adrenal hypertrophy in ascorbic acid pretreated animals exposed to cold. Observation of the effects on adrenal cholesterol levels led to the postulation of an indirect effect of ascorbic acid on adrenocortical hormone synthesis by increasing the adrenal cholesterol content. Bacchus & Toompas (261) have found that pretreatment with 150 mg. of sodium ascorbate does not prevent the lymphopenia induced by epinephrine in female rats, but histological changes have been observed in the adrenals of saline controls, which are not found in the pretreated rats. Furthermore, the latter showed eosinophilia whereas eosinopenia was found in the controls, suggesting to these workers a relationship of ascorbic acid to eosinophiles. Later, Bacchus *et al.* (262) found that the eosinophile response to ACTH,[2] however, was not prevented by ascorbic acid pretreatment in male albino mice, that the hematologic response to ACTH was, in fact, prolonged by pretreatment. This action of ascorbic acid was postulated to be the consequence of blocking the elaboration of ACTH, by reducing the degradation of corticosteroids. Stepto *et al.* (263) have made a study of the changes of adrenals and blood of guinea pigs on various levels of ascorbic acid intake after 7, 14, and 21 days from the beginning of the experiment. Statistically significant changes in eosinophiles, lymphocytes, and serum sodium were not noted. Decreases in adrenal ascorbic acid and slower decreases in adrenal cholesterol were found with the development of compensatory hypertrophy when the ascorbic acid-cholesterol ratio exceeded 1:500 (normal value 1:200).

Carbohydrate metabolism.—Altered carbohydrate metabolism in scurvy is well established, although little is known regarding the fundamental mechanisms involved and whether or not the effects are specific. Banerjee & Deb (264) consider that a combined deficiency of cortical hormones and insulin may be the cause of the derangement. Stewart *et al.* (250) have indicated, however, that insulin has little effect on the blood glucose of scorbutic monkeys in doses which are very effective in normal animals, and Murray (265) has found that neither adrenal cortical extract, insulin, nor epinephrine has any corrective influence on carbohydrate metabolism in scorbutic guinea pigs. Furthermore, McKee *et al.* (266) have found both glycogenolytic as well as gluconeogenetic functions impaired in scorbutic guinea pigs and could not

obtain a stimulating effect of cortical hormones on the production of liver glycogen. Apparently, the factors have not been used together although some glycogen deposition might be expected in such experiments, because of mass action alone. On the other hand, Bacchus *et al.* (267) have found a potentiating effect of large doses of ascorbic acid on the gluconeogenesis induced by cortisone in adrenalectomized mice. From other studies, it would seem that ascorbic acid can not only potentiate cortisone induced glycogen deposition, as shown by Bacchus *et al.* (267), but also potentiates the diabetogenic effect of alloxan [Patterson (268)], apparently diametrically opposing results. Ascorbic acid was less efficient, in this respect, than was DHA.[3] DHA alone, in fact, was found to be capable of producing diabetes, as was dehydroisoascorbic acid. Certain structural similarities between alloxan and DHA or its analogue were held responsible for their parallel action. Injections of ACTH are also known to produce diabetes mellitus, which may be more or less temporary, depending on the size of the dose and duration of the treatment. It will not be possible to go into the details of this work here. It is sufficient to mention the studies of Conn *et al.* (269) who found a close correlation between heightened uric acid production, as evidenced by increased uric acid excretion, and glycosuria in man, following injections of ACTH. The resemblance of uric acid to alloxan, and also to DHA, is shown in Figure 2.



Alloxan Uric acid Dehydro-L-ascorbic acid

FIG. 2. Structural relationships of uric acid.

Conn *et al.* (269), Patterson *et al.* (270, 271), and Banerjee *et al.* (272) believe the low glutathione levels encountered in their experimental animals to be responsible for depressed insulin secretion. Patterson *et al.* (270) have proposed, mainly on the basis of spectrophotometric evidence, that alloxan interacts with glutathione and sulfhydryl-bearing proteins to form addition products and that such a mechanism, i.e., the inactivation of essential sulfhydryl groups, could explain the irreversible inactivation of essential sulfhydryl enzymes. Subsequently (271), they showed that the nearly simultaneous administration of cysteine, glutathione, or 2,3-dimercaptopropanol with diabetogenic doses of DHA completely prevented any ill effects in all of 32 rats so treated while 13 of 15 unprotected rats became diabetic. These

compounds, if injected 10 minutes after the administration of DHA, apparently afforded no significant protective action, since 15 of 20 animals became diabetic. The authors concluded that diminished insulin secretion in the scorbutic guinea pig is caused by diminished stores of cysteine produced, in turn, by diminished glutathione levels or because the insufficient amounts of glutathione could no longer adequately inactivate DHA. Hence, injury to pancreatic β-cells or the sulfhydryl enzymes of β-cells might result. Banerjee *et al.* (272) did show increased DHA concentrations in scorbutic guinea pigs as compared with pair-fed control animals. However, the existence of high DHA levels in scorbutic guinea pigs has not been confirmed by the work of Damron *et al.* (273). An enzymatic, rather than hormonal disturbance is indicated by the results of Murray (274). Depressed phosphorylase activity was noted in the livers of scorbutic guinea pigs, but not in the kidneys, while adenosine triphosphatase activity was higher in the liver tissue of deficient animals. Increased oxygen consumption by deficient liver was also found under the conditions used. Patterson (275) has found that in adrenalectomized DHA-diabetic rats the substitution of 0.8 per cent sodium chloride for water reduces the symptoms of diabetes markedly while in nonoperated rats all the symptoms are increased except hyperglycemia, which diminishes.

Biosynthesis and metabolism.—The biosynthesis of ascorbic acid in the albino rat has been studied with labeled glucose thus providing the first direct evidence that carbohydrates may serve as precursors of this vitamin. Previous information, based primarily on increased excretion of ascorbic acid (reducing substances) in response to administration of suspected precursors, suffered either from lack of specificity or was questionable as a result of the well known stimulating action of substances that could not furnish the carbon skeleton (276). Using uniformly labeled glucose as the precursor, Jackel *et al.* (276) have concluded that the isolated ascorbic acid was labeled uniformly within the limits to be expected. While this did not preclude intermediate fission and recombination without differential dilution, a direct conversion of the glucose chain to ascorbic acid was held possible. Supporting evidence came from the studies of Horowitz *et al.* (277). Glucose labeled in the first carbon only was found to be converted to ascorbic acid possessing a preponderance of activity in carbon-six, with complete degradation showing a distribution of the remaining activity through the other positions. However, more recent evidence (255) indicates that the process is probably not so simple as pictured above. Carboxyl-C^{14}-labeled acetate (which gives rise to liver-glycogen glucose, labeled predominantly in carbons-three and -four) was converted to ascorbic acid which, as might be expected, was most heavily labeled in carbons-three and -four of ascorbic acid. However, under the conditions of these experiments, the carbon-four, -five, -six portion of the ascorbic acid carbon chain was found to be consistently more heavily labeled than the carbon-one, -two, -three portion. Corresponding results were obtained with uniformly labeled glucose. An intermediate cleavage into nonidentical three-carbon fragments must thus be considered possible, since glycogen

glucose in these same experiments showed no correspondingly nonsymmetrical labeling. A condensation product of glucose and acetoacetic acid, a possible ascorbic acid precursor in the pea, has been reported, but if correct, it must differ fundamentally from any process described above [Nath *et al.* (278)]. The use of labeled ascorbic acid has led to the clarification of certain gross aspects of the metabolism of the vitamin. Burns *et al.* (279) have shown the conversion of ascorbic acid into respiratory carbon dioxide and urinary oxalate by the guinea pig. The former constituted the main catabolic pathway, usually accounting for approximately 25 per cent of the administered dose within 24 hours, while oxalate contained less, although important amounts, of the radioactivity. Thus, the suspected formation of oxalate from ascorbic acid was demonstrated *in vivo*. Feces contained little radioactivity, possibly arising from urine contamination, and gastrointestinal washings were not radioactive. The distribution of radioactivity, moreover, corresponded closely to the distribution of the vitamin in various tissues. Significantly, chondroitin sulfuric acid and collagen were found to contain no radioactivity, and the C^{14} content of skin and nasal septa was extractable with 8 per cent acetic acid. By carrier experiments, the radioactivity of the nasal septa was shown to be unchanged ascorbic acid. The ascorbic acid content of the incisor teeth, as judged by radioactivity present, was found to be about four times the amount present in the adrenal glands when calculated on a per milligram of carbon basis. This is of interest when the important function of ascorbic acid in maintenance of normal tooth structure is considered. In further studies with the labeled vitamin, it was found that all radioactivity, within experimental error, could be accounted for as unchanged ascorbic acid in blood, livers, spleens, kidneys, and adrenals of normal guinea pigs, and adrenals of rats [Salomon (255)]. DHA[2] and reduced ascorbic acid were indistinguishable by the methods used, but accounted for essentially all the radioactivity. The necessarily low levels of DKA present concur with the findings of Damron *et al.* (273). Evidence for the reduced catabolism of ascorbic acid in scorbutic guinea pigs, ACTH-treated guinea pigs and rats, and guinea pigs treated with lethal doses of diphtheria toxin was found, as regards respiratory carbon dioxide. Urinary excretion of total radioactive end products did not change significantly in the limited number of experiments. Adrenal levels of radioactivity in ACTH-treated guinea pigs, and normal and hypophysectomized rats, could be correlated with ascorbic acid levels. Hence, depressed vitamin levels under "stress" conditions reflect the outward migration of ascorbic acid. This, in addition to the work of Vogt (280), would seem to shed further doubt on the findings of Lowenstein & Zwemer (281) on the existence of ascorbic acid-steroid conjugates.

Rats, which do not require exogenous vitamin C, were found to respond to exposure to cold by a considerable increase in excreted DKA,[2] DHA, and reduced ascorbic acid, in the order mentioned [Monier & Weiss (253)]. Whether the conclusion is justified that some cold-adaptation mechanism

utilizes the reducing capacity of ascorbic acid and that DKA and DHA are normal metabolic products in the rat, does not seem certain. It would have been of interest to determine the effect of cold on the excretion of oxalic acid, i.e., to what extent the breakdown of excreted ascorbic acid was prevented by low temperatures. Guinea pig urine is normally basic, an unsuitable medium for ascorbic acid, until it reaches the acid-containing collection unit.

Todhunter *et al.* (282), studying the utilization of DHA by human subjects, have determined the reduced and total ascorbic acid in blood and urine of seven healthy young women who ate a diet low in ascorbic acid, supplemented with 65 mg. of ascorbic acid or DHA. No differences in the utilization of the two forms of the vitamin could be found, based on reduced ascorbic acid values determined. Similar results have been obtained by De Ritter *et al.* (283) in seven male subjects. The antiscorbutic properties of 3-methyl-L-ascorbic acid and 1-methyl-heteroascorbic acid have been investigated [Goldman & Gould (284)]. No antivitamin properties were discerned. On the contrary, both substances exhibited more or less considerable antiscorbutic action. D-Glucoascorbic acid, constituting 5 or 10 per cent of the diet of rats or Syrian hamsters, did not produce the signs or symptoms of scurvy in a study by Shafer (285). Other effects, as diarrhea and reduced rate of growth, were noted after the addition of such amounts of the vitamin analogue. Rats and Syrian hamsters do not require vitamin C supplementation, but in guinea pigs, which do, a diet containing 5 per cent D-glucoascorbic acid plus 5 mg. of ascorbic acid per day did not induce onset of scurvy. In fact, a diet free of ascorbic acid but containing 5 per cent D-glucoascorbic acid, seemed to delay the appearance of scurvy or reduce the intensity. However, Sokoloff *et al.* (286), on the theory that D-glucoascorbic acid interferes with the synthesis of ascorbic acid, placed AK leukemic mice on a "scorbutogenic" diet containing 0.5 per cent flavonoids, and 5 per cent D-glucoascorbic acid which was subsequently reduced to 3 per cent, and then 2 per cent. It was stated that the plasma ascorbic acid levels fell to 0.03 to 0.05 mg. per 100 ml., and the white blood cell count to an average of 2500. Maintained on this regimen for several weeks without ill effects, the mortality of treated mice was said to be remarkably reduced over that of the untreated animals. It seems doubtful that the addition of D-glucoascorbic acid did actually interfere with ascorbic acid synthesis. More likely, some more or less nonspecific pharmacodynamic effect was observed.

Lowry *et al.* (165) have studied the effect of prolonged high ascorbic acid dosage in one female and three male subjects. One gm. of ascorbic acid per day for three months produced no harmful consequences of any kind, nor any progressive alteration in the tolerance curves, urinary excreton, serum, or white cell levels of the vitamin.

Rodahl (287) has confirmed the production of a condition resembling scurvy in guinea pigs on adequate diets, and in rats and dogs, by the administration of toxic doses of vitamin A. Furthermore, toxic doses proved more

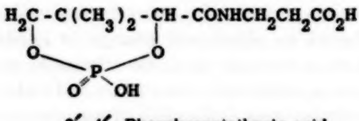
deleterious to scorbutic guinea pigs than to normal animals. Morehouse *et al.* (288) have found no significant change in hepatic ascorbic acid in hypovitaminosis A in rats under their experimental conditions, but noted the appearance of some scurvy-like symptoms and reduced hepatic ascorbic acid levels in hypervitaminosis A. Feeding of ascorbic acid supplements was, however, of no apparent benefit. Therefore, any specific relationship appears questionable, as is also indicated by the work of Robertson & Cross (289) on collagen formation in vitamin A deficient rats. The results of Eaton *et al.* (290), using vitamin A-depleted calves, may be interpreted in a similar fashion.

The depletion of adrenal ascorbic acid following ACTH injections has been the basis for speculations on the interrelationship with hormone synthesis, but a number of investigators have concluded that no such effect exists. It is of interest in this connection that the ascorbic acid levels in the adrenals of the chick [Jailer & Boas (291)] and quail [Zarrow & Baldini (292, 293)] do not change when ACTH is given, although other evidence, e.g., adrenal hypertrophy, seems to indicate that ACTH does stimulate the glands. Confronted with such evidence, it is tempting to arrive at definite opinions. Nevertheless, whether or not ascorbic acid is actually involved in the formation or release of adrenal cortical hormones can certainly neither be definitely shown, on the one hand, by a decrease of adrenal ascorbic acid following ACTH injection or "stress" in the mammal nor, on the other, by failure to find such a change under parallel conditions in the bird.

Pantothenic Acid

Recent progress in the investigation of pantothenic acid and its functional form, coenzyme A, is consistent with the central position of "active acetate" in metabolic processes.

Chemistry.—The structural characteristics of acetyl-CoA (coenzyme A), as reviewed by Welch & Nichol (1) and Ochoa & Stern (294), have received substantial confirmation from numerous sources. Wang *et al.* (295) have shown that the monoester phosphate group from the ribose moiety of CoA is removed by barley *b* nucleotidase. Proof of the structures of adenylic acid *a* and *b* has been given by Brown & Todd (296) and Reichard *et al.* (297). Baddiley & Thain (298) have identified 4′ and 2′,4′ phosphates in the alkaline hydrolysate of CoA. A third unidentified phosphate was probably the 2-mercaptoethylamide derivative of 2′,4′-phosphopantothenic acid. Pure pantetheine has been prepared in good yield by adaptation of the Bergman peptide synthesis [Baddiley & Thain (299)]. Recently, Baddiley *et al.* (300) have synthesized pure D(+)-pantetheine-4′-phosphate and shown that it is converted in 82 per cent yield to CoA by *Acetobacter suboxydans.* Forty-seven per cent of synthetic DL-pantetheine-4′-phosphate was found to be transformed into CoA by a partially purified enzyme system from pigeon liver. Gregory *et al.* (301) have isolated 93 per cent pure CoA from *Streptomyces fradiae.* The analysis of this sample confirms the current concepts regarding



2′, 4′-Phosphopantothenic acid

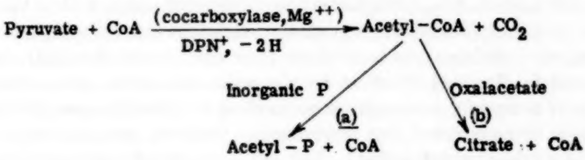
the composition of CoA. A method has been described for the preparation of CoA of high purity from dried brewer's yeast [Beinert *et al.* (302)]. The method, involving coprecipitation of eluates of CoA with glutathione and cuprous oxide, is adaptable to the preparation of relatively large amounts of CoA. The chief impurity found was glutamic acid [Beinert *et al.* (303)].

A simple acetylation of CoA with thiolacetate in alkaline solution has been achieved by Wilson (304). Acetic anhydride and isopropenyl acetate were less efficient acetylators. Nachmansohn *et al.* (305) have demonstrated that thiolacetate can be substituted for ATP (adenosinetriphosphate) and acetate in the acetylation of choline and sulfanilamide by a pigeon liver enzyme "acetylkinase" containing added CoA and squid extracts. Thiolacetate replaced ATP and acetate in the presence of transacetylase. The complete enzyme system, without CoA added, catalyzed the acetylation of cysteine and mercaptoethanolamine by thiolacetate. In the latter case, acetylation with the thiol group was established. Equilibrium data would be most desirable for estimation of the bond energy of thiolacetate. Brown & Snell (306) have been unable to detect any unidentified LBF (*Lactobacillus bulgaricus* factor) fragments in preparations treated with intestinal phosphatase. Purified CoA yielded fewer compounds with LBF activity than did similarly treated preparations of lesser purity. Pantethine and several forms of LBF were transformed into the same chromatographic CoA component by treatment with a sulfhydryl compound followed by reoxidation with iodine. Purer preparations of CoA contained only one sulfur atom per pantothenic acid residue. Gregory & Lipmann (307) have synthesized β-mercaptoethylamine disulfide from CoA preparations by acid hydrolysis. CoA might contain a single component of sulfur, presumably β-mercaptoethylamine linked through a disulfide bond to cysteine. Schwyzer (308) has treated pantetheine with ethylchloroacetate and vinylamine in triethylamine and condensed the product with thiolaceatate or thiolbenzoate. Both products yielded LBF on hydrolysis.

Biochemistry.—The important role of the thiol moiety in CoA function has been illuminated further through the quantitative equilibrium studies of the condensation of acetyl-CoA and oxalacetate to yield citrate and CoA in the presence of "crystalline-condensing enzyme." By coupling of the reaction with malate, DPN (diphosphopyridine neucleotide), and malic dehydrogenase, it was possible to determine the equilibrium constant for the condensing reaction. A bond energy for acetyl-CoA of 12,400 calories was obtained. For each equivalent of CoA liberated, one equivalent of

—SH group was formed. The affinity of acetyl-CoA for the condensing enzyme was high [Stern *et al.* (309)]. The acetylation of choline by active acetate has been coupled with several other enzymatic oxidation-reduction reactions. These included the reactions catalyzed by lactic dehydrogenase, malic dehydrogenase, and glucose dehydrogenase that function to reoxidize the DPN[2] required in the acetylation of CoA by pyruvate [Korkes *et al.* (310)]. Sanadi & Littlefield (311) have demonstrated that α-ketoglutamic oxidase catalyzes the following reaction: α-ketoglutarate$+DPN^+ +$CoA $\rightarrow$succinyl CoA$+CO_2+DPNH+H^+$. The enzyme, succinyl CoA deacylase, which catalyzes the reaction, succinyl CoA$+H_2O\rightarrow$succinate$+$CoA, has been described by Gergely *et al.* (312). The suggestion of Lynen *et al.* (313) that the acetylation of CoA possibly proceeds through the formation of a phosphorylated coenzyme, RSH_2PO_3, received confirmation with the isolation of CoA pyrophosphatase by Lipmann *et al.* (314). In the presence of a protamine, and an ammonium sulfate fraction of yeast extract, and fluoride, acetyl-CoA exchanges acetate for the pyrophosphate of ATP: ATP$+$CoA $\rightleftarrows$adenosinemonophosphate$+$CoA-pyrophosphate; CoA-pyrophosphate $+$acetate$\rightleftarrows$acetyl-CoA$+$pyrophosphate. The reaction favors the formation of acetyl-CoA. CoA-pyrophosphate was acid stable at room temperature and heat stable in neutral solution. The same series of reactions were found to occur with extracts from pigeon liver.

Littlefield & Sanadi (315) have identified at least four cofactors involved in the oxidation of pyruvate, namely, DPN, CoA, Mg^{++}, and cocarboxylase.



Phosphotransacetylase (*a*) increased the rate of oxidation. Triphosphopyridine nucleotide could not be substituted for DPN and pantetheine did not replace CoA. Protogen B, α-lipoic acid, and a boiled extract of α-ketoglutaric oxidase containing protogen, were inactive. Soluble enzyme preparations from pig heart and *Escherichia coli* [Korkes *et al.* (316)], with added DPN and CoA, catalyzed the dismutation of pyruvate to acetyl phosphate or citrate, if (*a*) bacterial phosphotransacetylase or (*b*) Ochoa's condensing enzyme were present. The system could be coupled to the malic dehydrogenase or glutaric dehydrogenase system. The similarities of the coenzyme requirements for pyruvate and acetaldehyde oxidation have been compared by Barner & O'Kane (317). *Proteus vulgaris* pyruvate oxidase did not require CoA and acetyl compounds did not accumulate; however, the formation of citrate and acetyl phosphate, and the acetylation of sulfanilamide indicated formation of at least traces of acetyl-CoA [Moyed & O'Kane

(318)]. CoA and DPN had no effect on the oxidation of pyruvate by pyruvate oxidation factor in the presence of the electron acceptors, ferricyanide or oxygen [Schweet & Cheslock (319)]. CoA was required for the acetylation of sulfanilamide by the pyruvic oxidase system. McLennan & Elliott (320) have not observed any effect of CoA on the synthesis of acetylcholine by brain slices or cell free brain preparations.

The relationship of thiol groups to the oxidation of fatty acids has been reviewed by Glass (321) and Lardy (322). Brady & Gurin (323) have demonstrated the incorporation of C^{14}-acetate into long chain fatty acids by a completely water soluble enzyme system. Similar incorporation of C^{14}-acetate into cholesterol did not occur [Brady & Gurin (324)]. The formation of P^{32}-containing phosphatidic acids from P^{32} labeled α-glycerophosphate and long chain fatty acids (stearic, oleic) was catalyzed by a partially purified enzyme from rat liver. The system requires ATP, CoA, and fatty acid. The formation of stearyl-CoA was postulated [Kornberg & Pricer (325)].

The existence of adrenal cortical lesions in pantothenic acid deficient rats has been correlated with impaired ability to secrete adrenal steroids, particularly the C-11 oxysteroids [Krehl *et al.* (326); Winters *et al.* (327)]. However, the survival of adrenalectomized rats was greatly prolonged when large amounts of pantothenic acid were added to the diet [Ralli (328)]. Kuhl *et al.* (329) have demonstrated, in young men exposed to cold water at 9.5°C., that pantothenic acid increases the blood ascorbic acid; cholesterol esters were lowered to a greater extent than was free cholesterol. These effects are increased on administration of pantothenate. It has been noted by Guehring *et al.* (330) that pantothenate deficient rats fed a high cholesterol diet are more resistant to fatty liver than are normal animals; also, that cholesterol feeding has no effect on the resistance of the pantothenate deficient rats to anoxia, as measured by increase in liver glycogen. Hurley *et al.* (331) have demonstrated that pantothenate deficient rats respond to anoxia as do adrenalectomized animals, but when given adrenal cortical extract before the test period, storage of liver glycogen reverts to normal. Although adrenal weight was increased, ascorbic acid content was low in pantothenate deficient animals.

Miscellaneous studies.—A statistically significant decrease in the CoA content of folic acid deficient chicken livers and sulfanilamide treated rats not receiving folic acid has been reported by Popp & Totter (332). In contrast to the chicks and sulfanilamide treated rats, rats receiving folic acid antagonists showed an increase in liver CoA.[3]

Growth of wild type *E. coli*, inhibited by salicylate, was reversed by pantoate and pantothenate. The pantothenate-synthesizing enzyme extracted from dried *E. coli* required ATP[2] and was not inhibited by low concentrations of salicylates. Inhibition at high concentration of salicylates was not reversed by pantoate [Maas (333)]. This enzyme was further purified. Acetyl phos-

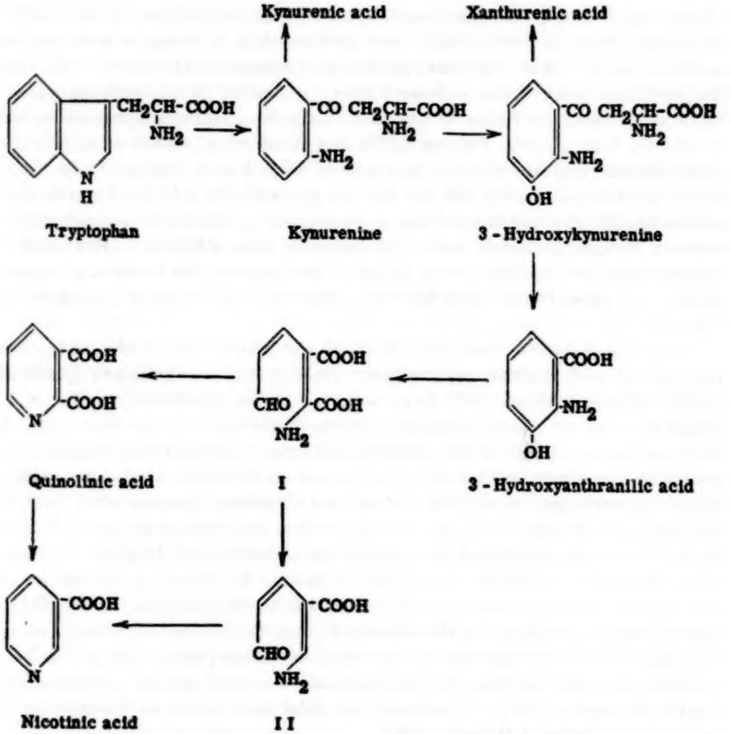
phate was formed, and the reaction was CoA independent [Maas (334)]. Recently, Maas & Davis (335) have succeeded in isolating a temperature sensitive mutant of *E. coli* that requires pantothenate only above 30°C. Cell free extracts of the mutant showed that the pantothenate-synthesizing enzyme itself was heat labile, in contrast to the heat stable enzyme extracted from wild type *E. coli*. Pomper (336) has obtained a mutant in which the pantothenate requirement can be replaced with *p*-aminobenzoic acid. Synthetic pantonyl-β-alanine did not replace pantothenic acid for *Lactobacillus arabinosus* growth and showed the same activity as did β-alanine and pantonine in reversing cysteic acid and salicylic acid inhibition. Pantonyl-β-alanine could replace pantonine in an *E. coli* mutant but could not replace pantoic or pantothenic acid for two other *E. coli* mutants [Lansford & Shive (337)].

The role of pantothenic acid in antibody production of the albino rat immunized with human erythrocytes appears to be significant [Axelrod (338)]. Allison & Wase (339) have observed an increased conjugation of 2-amino-fluorene on administration of pantothenic acid to deficient dogs. A study of the pathology of the intestine and other organs of weanling pigs on a diet low in pantothenate revealed excessive discharge of mucus, ecchymotic hemorrhages, superficial ulcers, and diarrhea [Sharma *et al.* (340)]. Pantothenate deficiency in the rat may lead to abortion or defective litters. Toxemia was not developed on ω-methylpantothenic acid. High levels, however, resulted in complete resorption of litters. By reducing the intake of the vitamin analogue, defective litters could be carried to term [Lewis (341)]. Pantothenate, employed with vitamin E, has been reported successful in the treatment of several cases of lupus erythematosus [Welsh (342)]. Alcoholics with delirium tremens did not respond to large doses of pantothenate [Smith & Brown (343)]. *Mycobacterium phlei* was shown to be capable of synthesizing citrate [Blakley (344)].

Methods.—Loy (345) has compared the results from eight different laboratories on the same pantothenate samples (346). The reduction of DPN by α-ketoglutaric acid catalyzed by its soluble oxidase and an auxiliary enzyme (deacylase) was CoA dependent and was used for analytical purposes. By this method, commercial glutathione was shown to contain 0.02 to 0.2 per cent CoA [Von Korff (347)]. The pantothenic acid content of fresh and 12-months cold storage eggs has been compared by Evans *et al.* (348).

NIACIN

Biosynthesis.—Some new knowledge has appeared concerning the steps involved in the biosynthesis of nicotinic acid from tryptophan, since the last review of this subject. Bonner & Yanofsky (349) have recently summarized the status of the subject in an unusually concise, critical, and orderly manner. The principal stages in the transformation appear to be as follows:



While many of the steps proposed above result from studies with mutant strains of *Neurospora*, many of them have been confirmed as occurring in animals also. In the absence of evidence indicating otherwise, it is useful to assume that this scheme describes the mechanism in animals. However, that this may not be true for other organisms should be kept in mind [Rhuland & Bard (350)]. Likewise, it should be kept in mind that a number of intermediate steps not shown nor yet known are probably involved. Compounds I and II are hypothetical intermediates; all other compounds indicated have been shown to support growth in the rat or to lead to the expected excretory products as well as supporting growth in *Neurospora*. This constitutes the principal evidence for the correctness of the scheme. Until recently, it appeared, on the basis of growth tests, that the rat could not use kynurenine to synthesize nicotinic acid. However, on the basis of isotope studies and the fact that rats excrete increased amounts of N^1-methylnicotinamide when given kynurenine, this seems not to be the case. The explanation for the failure

of growth response in the rat when given kynurenine may lie in the lack of stability of this compound and, therefore, a failure for it to reach the functioning site. As Bonner & Yanofsky (349) point out, "Experiments employing labeled kynurenine, with the subsequent isolation of niacin or N^1-methylnicotinamide, would go a long way toward clearing up this dilemma." There are enzymes present in several animal species that convert tryptophan to kynurenine. 3,4-Dihydroxyanthranilic acid has been suggested as a logical intermediate subsequent to ring cleavage. However, since this compound has been found inactive in rats, *Neurospora*, the mold *Xanthomonas pruni*, and in liver enzyme preparations that can convert 3-hydroxyanthranilic acid to quinolinic acid, the correctness of this suggestion is in doubt [Henderson *et al.* (351); Hellmann & Wiss (352)]. Cleavage of the six membered ring, with subsequent closure to include in the pyridine ring of nicotinic acid the nitrogen originally in the five membered ring of tryptophan, is required by the isotope evidence available [Bonner & Yanofsky (349); Partridge *et al.* (353)]. However, the details of this aspect of the scheme are not yet revealed. The alternate pathway through quinolinic acid is made necessary by the fact that although this compound relieves niacin deficiency in the rat and in *Neurospora*, it is far less effective than would be expected if it were a direct intermediate in the process. The intraperitoneal injection of tryptophan or of 3-hydroxyanthranilic acid increased the amount of quinolinic acid excreted by the rat, and enzyme preparations were found that convert 3-hydroxyanthranilic acid to quinolinic acid. Although there can be no question that quinolinic acid is closely related to tryptophan metabolism, at present this relationship is not clear. Bokman & Schweigert (354) have found spectrophotometric evidence for the appearance of an unidentified new compound capable of producing quinolinic acid when 3-hydroxyanthranilic acid is incubated with liver enzyme preparations. Perhaps this is the hypothetical intermediate, I. Distribution of this enzyme system in the rat is reported. The enzyme is present primarily in the liver and kidney, absent in muscle, spleen, and pancreas [Priest *et al.* (355)]. De & Guha (356) report that liver and kidney slices incubated in a medium containing different sources of nitrogen possesss the capacity to convert L-tryptophan into nicotinic acid. Ammonium sulfate was a satisfactory source of nitrogen; nitrate was not. Pyridoxine and folic acid accelerated this *in vitro* conversion; thiamine did not. Iron, copper, and manganese were likewise stimulatory. The analytical methods used did not exclude the possibility that quinolinic acid, not nicotinic acid, was the end product measured. Since stimulation was found with so many substances, confirmation of the work would seem desirable.

Partridge *et al.* (353) conclude that niacin is synthesized solely by way of tryptophan in *Neurospora*. The larvae of *Tenebrio molitor* and *Tribalium confusum* can not synthesize niacin from tryptophan, according to Fraenkel & Stern (357). The nicotinic acid content of pulses doubles during the first four days of germination. This is not true for all germinating seeds [Banerjee *et al.* (358)]. Feigelson *et al.* (359) find that tryptophan is equally as effective

as nicotinic acid and as nicotinamide in immediately increasing the pyridine nucleotide concentration in the livers of rats previously made deficient in niacin-trypophan. In the event that protein is also low in the basal diet, tryptophan may be superior in this respect, especially in older animals [Williams *et al.* (360)].

Radioautographs prepared from paper-strip chromatograms of the urine from rats that previously received either nicotinic acid or nicotinamide (C^{14}-labeled carboxyl) showed the presence of seven metabolic products when nicotinic acid was used and nine when nicotinamide was used. Four of the products were identified as N^1-methylnicotinamide, nicotinuric acid, nicotinic acid, and nicotinamide [Johnson & Lin (361)]. The unidentified compounds represented approximately 10 and 20 per cent respectively of the total activity excreted. Leifer *et al.* (362) in similar experiments with mice, rats, hamsters, and dogs have obtained six bands. In addition to the compounds identified above, they have found N^1-methyl-6-pyridone-3-carboxylamide. The unidentified band was not trigonelline. Some C^{14}-carbon dioxide was found in the expired air of the species examined. Hughes (363) has shown that 6-hydroxynicotinic acid is an intermediate in the oxidation of nicotinic acid by *Pseudomonas fluorescens* to carbon dioxide plus water and points out that oxidation in the sixth position is the first step in the oxidation of a benzene ring by many organisms.

In the human adult, there is a marked increase in the urinary excretion of N^1-methylnicotinamide that is progressive with pregnancy and remains for some time postpartum. Although there is considerable variation with individual subjects, the total excretion of nicotinic acid and known metabolic products equals or exceeds, in some cases, the sum of the niacin ingested and the niacin expected to be available from tryptophan. It is suggested that important factors other than niacin, tryptophan, and protein intake are involved. Similarly, the rat shows an increased excretion during pregnancy, but to a lesser extent [Lojkin *et al.* (364)]. Beher *et al.* (365) have observed that testosterone injections affect the rate of N^1-methylnicotinamide excretion in the rat and that the concentration and total amount of pyridine nucleotides in the rat liver are increased by this hormone. Cantoni (366) has obtained from the liver of the rat, pig, guinea pig, and dog a soluble enzyme preparation that, in the presence of L-methionine as a methyl donor, ATP, and magnesium ions, converts nicotinamide to N^1-methylnicotinamide. The preparation could not be obtained from the liver of the horse, sheep, ox, rabbit, pigeon, or duck. Elsewhere in this review, the effect of folic acid, citrovorum factor, and vitamin B_{12} on N^1-methylnicotinamide is discussed.

Studies of the tryptophan-niacin relationship have been made more complicated by the number of factors that affect the process. The addition of gelatin, zein, or certain amino acids, such as threonine or phenylalanine, to a low tryptophan-niacin diet accentuates the deficiency [Lyman & Elvehjem (367); Anderson *et al.* (368)]. A simultaneous deficiency of other vitamins also interferes with the conversion. While in some cases the effect may be non-

specific, the change in excretion of quinolinic acid observed by Henderson *et al.* (369) when certain intermediates are fed, indicates that riboflavin may be more specifically necessary for the conversion of kynurenine to 3-hydroxykynurenine and that pyridoxine may be essential for the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid (see vitamin B_6 section). The reason for the accentuation of the tryptophan-niacin deficiency by an imbalance of amino acids is not clear, and the cause may not be the same in all cases. Ebisuzaki *et al.* (370) have found that a threonine induced amino acid imbalance can be demonstrated in the rat when 9 per cent casein in the basal diet is fed as whole casein but not when the protein is supplied as a mixture of free amino acids simulating 9 per cent casein, and suggest that the threonine influences the availability of the amino acids in whole casein, possibly by inhibiting certain digestive processes. The previous observation that threonine given intraperitoneally will also produce this imbalance is not yet explainable by the above hypothesis. Porter *et al.* (371) indicate that the adrenal cortical hormones may also influence tryptophan metabolism.

Pellagra.—Goldsmith *et al.* (372) have produced clinical signs of pellagra in three subjects maintained for more than 50 days on a "corn" diet containing 4.7 mg. of niacin and 190 mg. of tryptophan in the daily ration. No evidence of pellagra was observed in one subject who received the "corn" diet for 42 days, in one subject who received 2 mg. additional niacin daily for 122 days, nor in a subject who received a wheat diet containing 5.7 mg. of niacin and 230 mg. of tryptophan daily for 95 days. The excretion of N^1-methylnicotinamide decreased to 0.5 mg. per day in those subjects showing signs of pellagra and was around 1 mg. per day in the others. Excretion of niacin, quinolinic acid, and tryptophan remained essentially constant throughout the experimental period, and the level of 6-pyridone fell to nondetectable levels after the first two weeks. The low excretion of niacin metabolites following administration of test doses of nicotinamide at the end of the basal period and the slow increase in excretion following therapy with nicotinamide or tryptophan, indicated depletion of body stores of niacin. All lesions responded to nicotinamide or to tryptophan. These findings suggest that the minimal niacin requirement of a man receiving 190 mg. of tryptophan daily is approximately 7 mg. per day. In the Rhesus monkey, Tappan *et al.* (373) have produced symptoms of niacin deficiency that respond to niacin or tryptophan. The conversion ratio of tryptophan to niacin in the monkey was found to be of the same order of magnitude as that for man and the guinea pig, i.e., 0.2 per cent. The lamb, calf, and pig show a conversion of about 1 per cent, and the rat 10 to 20 per cent. The chick, horse, and rabbit also convert tryptophan to niacin; however, the domestic cat appears to be unable to do so [Da Silva *et al.* (374)]. Oesterling & Rose (375) have found that while nicotinic acid stimulates the growth of rats receiving definitely suboptimum amounts of tryptophan, the absence of the vitamin from the food produces no measurable effect upon tryptophan requirements. Childs *et al.* (376) report that 2.8 mg. of niacin per 100 gm. of

food are required for rapid growth of the chick and that although tryptophan spares niacin to some extent, this does not entirely compensate for a partial niacin deficiency.

In Mexico, there is high consumption of corn but little pellagra. The corn is eaten as tortillas for which the whole corn is previously cooked in lime water. Laguna & Carpenter (377) have shown that corn prepared in such a manner, in contrast to raw corn, supports growth in niacin deficient rats, thus emphasizing previous observations that corn and other cereals contain niacin that is made available to the rat and probably to man by hydrolysis with weak alkali [Krehl (378)]. This suggests that such a method of food preparation may be a factor in the prevention of pellagra in this corn-eating population. That the full effect of the lime water treatment was obtained from the nonstarchy fractions of the corn, is explained by the distribution of niacin in the seed, which has been studied recently by Heathcote *et al.* (379). Ghosh *et al.* (380) report the presence in certain fish of a "bound form" of niacin that is different from that found in cereals. Reddi (381) has proposed another method for estimating the "bound form" of nicotinic acid. The niacin content of 88 Puerto Rican foods has been reported by Asenjo (382).

Pyridine nucleotides.—Diphosphopyridine nucleotide (DPN) plays an important role in the metabolism of most living cells. Most cells are able to synthesize and enzymatically destroy this nicotinamide-containing coenzyme. The reactions involved in this synthesis and destruction are rapidly being revealed. Kornberg (383) has obtained from yeast and liver a DPN-synthesizing enzyme preparation that catalyzes the reaction ATP+NMN (nicotinamide mononucleotide, nicotinamide ribose-5-phosphate)$\rightleftarrows$DPN+pyrophosphate. This enzyme has been found by Hogeboom & Schneider (384) to reside in the nucleus. This is interesting because other distribution studies indicate that most of the DPN-linked enzyme systems are largely confined to the cytoplasm. Rowen & Kornberg (385) have obtained an enzyme preparation which catalyzes the reaction: Nicotinamide +ribose-1-phosphate+$H^+$$\rightleftarrows$orthophosphate+$NR^+$ (nicotinamide riboside). In turn, the synthesis of NMN from NR^+ has been observed in the presence of ATP and crude liver fractions. Human erythrocytes, washed with saline, produce on incubation with 2 per cent nicotinamide an eight- to tenfold increase in cellular pyridine nucleotides; 75 to 90 per cent of the product was found to be NMN [Leder & Handler (386)]. When suspended in media containing glucose and nicotinic acid, *L. arabinosus* grown on niacin deficient media synthesizes cozymase (DPN plus triphosphopyridine nucleotide) until the cells contain a normal quantity; synthesis then ceases [Hughes & Williamson (387)]. The enzymes that split DPN are widespread and of at least two types, DPN nucleotidase that catalyzes the cleavage of the glycosidic bond between nicotinamide, and ribose and DPN pyrophosphatase that catalyzes the cleavage of the pyrophosphate bond between adenylic acid and nicotinamide mononucleotide. Nicotinamide specifically inhibits the nucleosidase and provides a simple way of distinguishing between these two modes

of DPN breakdown [Kornberg & Pricer (388)]. Alivisatos & Denstedt (389) have shown that the DPN nucleotidase of rabbit erythrocytes is confined to the exterior surface of the stroma. DPN is rapidly destroyed by suspensions of the cells unless nicotinamide is added to inhibit the enzyme. By protecting the DPN with nicotinamide during the preparation of the powder, Hochster & Quastel (390) have been able to prepare an acetone powder of yeast that retains its fermentative power for several months.

The observation that isonicotinic acid (4-pyridinecarboxylic acid) hydrazide and related compounds are chemotherapeutically effective in experimental and human tuberculosis has stimulated vigorous activity on the synthesis of new compounds of this type and on the study of their toxicity, effectiveness, and shortcomings [Bernstein *et al.* (391); Steenken & Wolinsky (392); Benson *et al.* (393); Rubin *et al.* (394); Robitzek & Selikoff (395); Elmendorf *et al.* (396); (397)]. It has become apparent that one of the limitations in the use of these chemotherapeutic agents will be the rapidity with which resistant strains of the tubercle bacillus arise [Steenken *et al.* (398); Buck & Schnitzer (399); Pansy *et al.* (400); Middlebrook (401); Szybalski & Bryson (402); Hobby & Lenert (403); (404)]. Of particular interest to the biochemist will be the mechanism of action of these nicotinic acid derivatives. Zatman *et al.* (405) have made observations on this point. Animal tissues contain an enzyme (DPN nucleotidase) that cleaves DPN at the nicotinamide riboside linkage to yield nicotinamide. Although this over-all reaction is irreversible, as indicated by the lack of enzymatic synthesis of DPN from nicotinamide, exchange studies have shown that enzyme preparations from certain tissues result in complete equilibrium of the unlabeled nicotinamide in DPN with labeled nicotinamide. However, preparations from other tissues containing DPN nucleotidase do not effect this exchange. Furthermore, it has been observed that, whereas isonicotinic acid hydrazide is approximately 10 times as effective as nicotinamide as an inhibitor of this over-all reaction in certain tissues, it is inactive in others. It is suggested that tissue synthesis of DPN containing the hydrazide instead of nicotinamide (a compound that likely would be biologically inactive) could be the mechanism sought, and that the greater sensitivity of the tubercle bacillus to isonicotinic acid hydrazide compared with the host is explainable by the above observations.

Vitamin B_6

The term, vitamin B_6, will be used as a group name to include pyridoxal, pyridoxamine, and pyridoxine, whereas the specific chemical names will be used only in reference to the corresponding compound [Snell & Wright (406)]. Vitamin B_6 occurs in nature primarily as pyridoxal or pyridoxamine, or their phosphate esters. However, since all three forms are of equal nutritive value for man and most animals, under the usual dietary conditions, synthetic pyridoxine has been the form most widely used in nutritional studies. Many microorganisms can not use pyridoxine, but require one of the

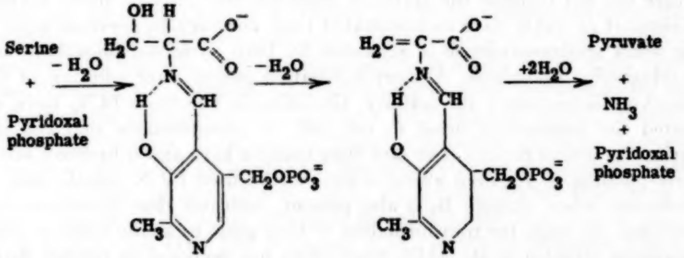
other forms. When mixed in synthetic diets high in soluble carbohydrates, pyridoxal and pyridoxamine are less active for the chick and rat than is pyridoxine. The presence of lactose or aureomycin increases the effectiveness of vitamin B_6 at low levels of intake. These effects are consistent with known changes in intestinal flora and indicate competition of intestinal flora with the host for limited supplies of the vitamin [Waibel *et al.* (407); Linkswiler *et al.* (408)].

Chemistry.—New methods for the synthesis of pyridoxine have appeared. Jones & Kornfeld (409) have avoided some problems of previous methods by introducing the hydroxymethyl group in positions four and five of the pyridine ring by reducing the methyl esters of the corresponding dicarboxylic acid with lithium aluminum hydride, and Jones (410) and Cohen *et al.* (411) have proposed methods based on the use of open chain compounds. Heyl *et al.* (412) have prepared additional pyridoxylamines, all of which are 50 to 100 per cent as active as pyridoxine in the rat, and Jones (413) has synthesized five vitamin B_6 analogues, two of which are weak antagonists. Desoxypyridoxine continues to be the most potent antagonist available and the one most widely used in experimental studies.

The synthesis of 3-pyridoxal phosphate, which proved to be biologically inactive [Heyl & Harris (414)], and the synthesis of 5-pyridoxal phosphate and 5-pyridoxamine phosphate, both of which are highly active and of proven structure, apparently settles the question of structure of codecarboxylase, cotransaminase, and a number of other pyridoxal phosphate-containing coenzymes [Heyl *et al.* (415); Wilson & Harris (416); Baddiley & Mathias (417); Viscontini *et al.* (418); Peterson *et al.* (419)]. Hurwitz (420) has obtained an enzyme preparation (pyridoxal-kinase) from brewer's yeast (by the usual methods of fractionation) that, in the presence of ATP,[2] catalyzes the phosphorylation of pyridoxal to pyridoxal phosphate. A divalent cation (Mg^{++}, Co^{++}, Mn^{++}, Ni^{++}, or Zn^{++}) is required and adenine, adenosine, adenylic acid, and inosinic triphosphate are competitive inhibitors. The enzyme phosphorylates other vitamin B_6 derivatives, provided the six-position of the pyridine ring is free and the five-position contains a hydroxymethyl group. Such compounds, e.g., desoxypyridine phosphate, competitively inhibit the union of codecarboxylase (pyridoxal-5-phosphate) with tyrosine apodecarboxylase.

Metzler & Snell (421) have studied the rates of the reversible transamination reaction, at 100°C., between pyridoxal and a number of amino acids, i.e., pyridoxal+glutamate$\rightleftharpoons$pyridoxamine+α-ketoglutarate. This reaction is catalyzed by copper, iron, and aluminum salts; the rates depend upon the particular amino acid; the pH optimum is about 4.5; and at equimolar reactant concentrations the equilibrium lies in most cases at about 50 per cent conversion to products. However, transamination between pyridoxal phosphate and glutamate is rapid under these conditions and goes nearly to completion. Baddiley (422) suggests that the phenolic aldehyde structure of pyridoxal should and does lead to the formation of metal co-ordi-

nation compounds between pyridoxal and amino acids and proposes a mechanism of electron displacement attributable to the formation of such a complex, which could explain the above transamination reaction. The significance of this proposal and the function of metals in biological transamination await the availability of purer enzyme preparations and studies under conditions more nearly physiological than are possible with these model systems. Baddiley also suggests that the union between pyridoxal phosphate and its various apoenzymes is through the phosphate group and the ring nitrogen. Reactions between serine or cysteine and pyridoxal, at 100°C., led to pyruvate and ammonia in one case and to pyruvate, ammonia, and hydrogen sulfide in the other [Metzler & Snell (423)]. On the basis of the similarities of pH optima and metal salts catalysis for these deaminations and the transamination reactions described above, Metzler & Snell (424) suggest that in the serine and cysteine reactions a transamination occurs first, followed by dehydration rearrangement and hydrolysis, to yield the observed products. The reactions are pictured as follows:



A Schiff base, perhaps stabilized by chelation with a metallic ion or a hydrogen bond is formed. By shift of the double bond and migration of the α-hydrogen, transamination can occur, or by the splitting out of water (or hydrogen sulfide) the Schiff base of aminoacrylic acid could be formed and undergo spontaneous hydrolysis to free aminoacrylic acid and then to ammonia and pyruvic acid. It is further suggested that the reaction of other substances across the double bond of the aminoacrylic acid moiety could be the mechanism for the formation of tryptophan from indole, and cystathionine from homocysteine and serine, all biological reactions known to require pyridoxal phosphate.

Pyridoxal-requiring enzyme systems.—Metzler & Snell (424) have prepared cell free extracts from *E. coli*, containing a pyridoxal phosphate-requiring D-serine dehydrase that rapidly converts D-serine to pyruvate. The enzyme is specific for D-serine and can be separated from L-serine dehydrase which is also present in dried cells. Cell free extracts of *Neurospora* contain a pyridoxal phosphate-requiring deaminase for L-serine and L-threonine [Reissig (425)]. Braunshtein & Goryachenkova (426) and Binkley *et al.* (427) have prepared extracts from rat liver containing pyridoxal phosphate-

requiring enzymes that catalyze the production of cysteine from serine and homocysteine. Binkley *et al.* (427) find that at least two enzymes are involved. *S. faecalis* requires either D-alanine or pyridoxal phosphate for growth. A cell free enzyme preparation (racemase) that converts L-alanine to D-alanine in the presence of pyridoxal phosphate has been obtained by Wood & Gunsalus (428). *L. arabinosus* contains a pyridoxal phosphate-requiring glutamate racemase which converts either D- or L-glutamic acid to a racemic mixture. Narrod & Wood (429) and Sinclair (430) conclude that pyridoxal phosphate is a coenzyme of histaminase and that the most probable explanation of the lesions of the skin, mucocutaneous junctions [Ramalingaswami & Sinclair (431)], and the priapism in vitamin B_6 deficiency is that they are secondary to vascular change produced by abnormal amounts of histamine. Roberts *et al.* (432) have found that in vitamin B_6 deficient rats the glutamic decarboxylase activity is about 50 per cent of normal. Refeeding with pyridoxine returned the activity to normal. The apoenzyme content was not diminished by the deficiency, and high levels of pyridoxine intake did not increase the glutamic decarboxylase activity above normal. Meister *et al.* (433) have demonstrated that, contrary to previous reports, pig heart apotransaminase is activated by both pyridoxal phosphate and pyridoxamine phosphate. A short incubation period after addition of the coenzyme is necessary for activity. Gunsalus & Tonzetich (434) have reported the presence in dried *E. coli* cells of transaminases that can use purines as amino donors. The fact that many α-keto and α-hydroxy acids, corresponding to required amino acids, can be used by *S. faecalis* and *L. arabinosus* when vitamin B_6 is also present, indicates that transamination furnishes the route for transformation of keto acids to amino acids in these organisms [Holden *et al.* (435)]. Snell (436) has reviewed in further detail the evidence showing the correlation between the chemical, enzymatic, and growth-promoting properties of vitamin B_6.

That vitamin B_6 is involved in tryptophan metabolism has been repeatedly demonstrated by the appearance of a number of the intermediate products of metabolism of this amino acid in the urine of deficient animals given tryptophan. These include kynurenine, 3-hydroxykynurenine, kynurenic acid, xanthurenic acid, N^{α}-acetyl derivatives of both kynurenine and 3-hydroxykynurenine, and conjugated derivatives of kynurenic and xanthurenic acids. Dalgliesh *et al.* (437, 438) point out that in vitamin B_6 deficient rats all of the 8 or 10 tryptophan metabolites that they have been able to separate from urine and identify by the use of chromatographic methods still contain the carbon atoms originally in the alanine side chain of tryptophan. Dalgliesh, therefore, suggests that vitamin B_6 must be concerned in the stage of metabolism of tryptophan at which the side chain is removed. This view is supported by the observation of Henderson *et al.* (369) that, following ingestion of tryptophan or kynurenine, vitamin B_6 deficient rats excrete much smaller amounts of nicotinic acid and quinolinic acid than do normal rats but that ingestion of 3-hydroxyanthranilic acid leads to a

normal excretion of these compounds. Mason & Berg (439) have shown that the decreased conversion of kynurenine to anthranilic acid and derivatives in liver homogenates from vitamin B_6 deficient rats is increased by the addition of pyridoxal phosphate. It was previously observed that the pyridoxal phosphate-requiring kynureninase in liver can also convert 3-hydroxykynurenine to 3-hydroxyanthranilic acid. As vitamin B_6 is known not to affect the conversion of tryptophan to kynurenine, it seems reasonably certain that Dalgliesh's suggestion as to the general nature and site of function of vitamin B_6 in tryptophan metabolism is correct (see Niacin section). Wachstein & Gudaitis (440) have found that pregnant women excrete more xanthurenic acid after a test dose of tryptophan than nongravid controls and that the administration of pyridoxine returns the excretion to normal levels. This observation supports the suggestion that the usual vitamin B_6 intake is inadequate during pregnancy.

Miscellaneous studies.—Sarett (441) has found the measurement of 4-pyridoxic acid, the principal urinary excretion product of vitamin B_6, useful in excretion studies following test doses of the vitamin, but of limited value in evaluating the dietary intake of vitamin B_6 directly. Substances other than 4-pyridoxic acid, which are present in normal urine and which can not be eliminated by dilution, lead to erroneously high results under these conditions. Schwartz & Kjeldgaard (442) have isolated pyridoxic acid as the end product of the reaction of liver aldehyde oxidase on pyridoxal. Antabuse inhibits the reaction. High protein diets, or increased methionine, homocysteine, or glutamic acid in the diet, aggravate vitamin B_6 deficiency in the rat. Tryptophan, tyrosine, serine, lysine, and cystine do not show such an effect [Cerecedo & De Renzo (443); De Bay *et al.* (444); Beaton *et al.* (445)]. Vilter & Schreiner (446) and Schreiner *et al.* (447) have observed that patients with natural seborrheic dermatitis respond to pyridoxine applied topically, but not to pyridoxine administered orally or parenterally. More than 90 per cent of vitamin B_6 deficient rats resorb their fetuses by midpregnancy, according to Nelson *et al.* (448, 449), as a result of inadequate ovarian production of estrone and progesterone. Inadequate secretion or production of pituitary gonadotropin and inadequate ovarian response to gonadotropins are also involved. The blood picture in vitamin B_6 deficiency in the monkey has been studied in detail by Poppen *et al.* (450). Rinehart & Greenberg (451) have described arteriosclerotic lesions in vitamin B_6 deficient monkeys.

Thiamine

Analytical methods.—A detailed report on the use of the thiochrome method for analysis of cereal, malt, yeast, and meat products, authored by a panel of chemists of the Society of Public Analysts and Other Analytical Chemists, has appeared (452), and an improved method for urine analysis has been described [Bessey *et al.* (453)]. A microfluorometric method for thiamine and its phosphates in blood and blood cells, which it is hoped will

be useful in evaluating nutritive status of populations, has been published by Burch *et al.* (454). Dubé *et al.* (455) have used the method to measure the blood thiamine content of human subjects maintained on controlled levels of the vitamin, and Burch *et al.* (456) have reported results of its use in nutritional surveys in the Philippines. Teeri (457) has found that the measurement of the fluorescence produced when cyanogen bromide and thiamine react is a suitable analytical method. Chromatographic methods have been useful in detecting and separating thiamine and its various phosphates and decomposition products [Cacioppo & La Grutta (458); Baldantoni *et al.* (459); Viscontini *et al.* (460); Rossi-Fanelli *et al.* (461)]. Such methods have led to the identification of triphosphothiamine in liver and have helped in the separation and study of the same compound prepared synthetically. Miller & Aschner (462) have developed an auxanographic method with yeast that is useful in detecting the presence of thiamine, cocarboxylase, and the thiazole and pyrimidine moieties of the vitamin. The method is semiquantitative and helpful in studying certain aspects of thiamine metabolism.

Occurrence and stability.—Because of the importance of wheat and bread as dietary sources of thiamine, several studies of the thiamine content of various types of wheat and bread have been reported from England, Holland, and Finland [Greer *et al.* (463); van der Mijll Dekker (464); Pulkki *et al.* (465)]. The distribution of thiamine and riboflavin in rice grains, the change in distribution of thiamine during the process of parboiling, and the retention of thiamine during the cooking of brown rice have been investigated [Simpson (466); Kennedy & Tsuji (467)].

One of the factors which must receive constant attention in the enrichment of flour is the stability of the materials added. It would appear from the studies of Hollenbeck & Obermeyer (468) that thiamine mononitrate is more likely to withstand adverse conditions under storage and transportation of flour than is thiamine hydrochloride. The lack of stability of thiamine when added to certain purified diets widely used for experimental purposes (probably caused by the presence of certain mineral salts) is emphasized by the observations of Lyman & Elvehjem (469). During the slow freezing or thawing of tissues, such as liver [Hammond (470)], thiamine pyrophosphate may be hydrolyzed by the phosphatases present.

Antagonists.—Interest continues in thiamine antagonists as a useful means of studying thiamine metabolism and as a possible means of controlling certain pathological processes. Oxythiamine, a compound in which an —OH group has been substituted for the —NH_2 group on the pyrimidine ring, and neopyrithiamine, a compound in which a pyridine ring has been substituted for the thiazole ring in thiamine, have received the most attention in this respect. Methods of synthesizing and testing the potencies of these compounds in the production of thiamine deficiencies in mice and rats have been described [Rydon (471); Cerecedo *et al.* (472); Emerson & Casey (473)]. Neopyrithiamine appears to be the more potent of the two com-

pounds and has an index of inhibition of the order of 5:1 in the rat. Woolley (474) has observed that neopyrithiamine inhibits the biosynthesis of cocarboxylase by chicken blood and that it does not inhibit the combination of cocarboxylase with the apocarboxylase. Velluz & Herbain (475) have shown by the use of washed yeast suspensions (source of carboxylase) that oxythiamine triphosphate strongly inhibits the functioning of the cocarboxylase system, but that free oxythiamine has no effect. It seems probable that these antagonists exert their effect at two points, i.e., by interference with the synthesis of cocarboxylase by the cell and by interference with the union of cocarboxylase with the apocarboxylase. The latter type of interference would be contingent upon the cell's ability to phosphorylate the antagonist. Although not demonstrated, this biosynthesis seems a likely possibility. Purified preparations of thiaminokinase that would be suitable for investigation of this point are now available (see below). Woolley & Merrifield (476) claim that the levels of cocarboxylase in the liver and of pyruvate in the blood are normal in animals dying with typical manifestations of thiamine deficiency produced by neopyrithiamine while these same substances are low and the symptoms less typical in those animals which succumb to the deficiency produced with oxythiamine. Therefore, it is suggested that these antagonists affect different systems and that it is not the low cocarboxylase but, perhaps, low levels of some other thiamine compounds that are responsible for the usual manifestations of thiamine deficiency. More evidence will be needed to establish this hypothesis.

Biosynthesis of cocarboxylase.—Knowledge of the mechanism whereby thiamine is transformed into one of its functioning forms (cocarboxylase, thiamine pyrophosphate) has been advanced by the preparation of an enzyme system (thiaminokinase) from rat liver and from yeast that, in the presence of ATP[2] and Mg^{++}, will synthesize cocarboxylase [Leuthardt & Nielsen (477); Steyn-Parvé (478); Chevillard & Thoai (479)]. The reaction has an optimum pH of approximately seven and requires a large excess of ATP. The reaction is inhibited by AMP[2] and ADP.[2] Only small quantities of cocarboxylase are formed when thiamine is replaced by its monophosphate. It is, therefore, supposed that the cocarboxylase is formed by the transfer of a pyrophosphate group from ATP to thiamine. However, Thoai *et al.* (480) claim to have obtained synthesis with their yeast preparation, using ADP in the absence of myokinase. This reaction was more rapid with thiamine monophosphate than with free thiamine. Chevillard & van Thoai (479) have found a number of similarities in yeast phosphokinase preparations that phosphorylate thiamine and pyridoxine. Both enzymes precipitate with ammonium sulfate at about the same concentration; the stabilities are alike and both can use either ATP or ADP for the phosphorylation. The presence of one of the vitamins inhibited the phosphorylation of the other, i.e., the phosphorylation of thiamine was completely inhibited by an equal quantity of pyridoxine. However, it required 10 to 20 times as much thiamine to inhibit phosphorylation of the pyridoxine. Smits & Florijn (481) have

shown that the phosphorylation of thiamine by erythrocytes is dependent upon glycolysis, and Leuthardt & Nielsen (477) observed that supernatants from liver homogenates required the presence of an active phosphorylating system (mitochondria plus oxidizable substrate) in order to maintain the synthesis of cocarboxylase. These systems supply the ATP needed for the phosphorylation in the living organism. The several reports indicating that the phosphorylation of thiamine is defective in diabetes mellitus is understandable on the above basis. It has been observed that the cocarboxylase content of the livers of diabetic rats does not increase in response to thiamine injection, whereas, that of normal animals and diabetic animals receiving insulin does [Siliprandi & Siliprandi (482)]. The cocarboxylase content of the blood of diabetic patients has been observed to return from a low level to normal within 30 minutes after insulin is given [Siliprandi & Navazio (483)]. Studies with liver homogenates from diabetic rats also indicate that in diabetes there may be a limitation in the rate of phosphorylation of thiamine [Foa *et al.* (484)]. Whether this means that the diabetic patient may be handicapped by a general lowering of the concentration or availability of ATP awaits further studies. Investigations of the distribution of thiamine pyrophosphate in rat liver fractions separated by a differential centrifugation procedure have shown that 90 per cent of the total thiamine pyrophosphate is divided equally between mitochondria and soluble fractions; the nuclei and microsomes contain a small amount [Goethart (485)].

Thiaminase.—The occurrence and properties of thiaminase, a thiamine-splitting enzyme found in the viscera of many fish and molluscs and in some bacteria and plants, has been well summarized in a recent review [Harris (486)]. Therefore, only the more recent publications on the subject will be referred to here. There is accumulating evidence that the disease which occurs in livestock consuming ferns and related species of plants, and referred to as bracken disease, is caused, at least in part, by the presence of a thiaminase in these plants. When the disease is not too far advanced, injections of thiamine usually lead to recovery. However, there are enough exceptions to this experience to lead to the suspicion that other factors may also be involved [Forenbacher (487); Lott (488); Moon & Raafat (489)]. Rats receiving sun dried bracken responded to thiamine by weight gain and although animals receiving autoclaved bracken were not completely normal, they were less affected than those receiving the untreated leaves [Cordy (490)]. Evans & Jones (491) have shown that extracts of bracken leaves contain a thermolabile factor (apoenzyme) that destroys thiamine and that *m*-aminobenzoic acid and 5-aminosalicyclic acid can replace a naturally occurring, heat stable coenzyme, which is also necessary for the destruction of thiamine by the bracken extract. Walsh *et al.* (492) have found that the injection of a partially purified extract of carp viscera, containing thiaminase, produces in mice a disjointed gait, temporary hind limb paralysis and loss of muscle tonus, a syndrome similar to that of thiamine deficiency. Thiaminase has been found present in seven additional species of fresh water fish and

absent from two. More data on distribution will be required to determine whether the distribution of thiaminase in fish is of phylogenetic significance [Chaet & Bishop (493)].

Krampitz & Woolley (494) previously isolated from carp-inactivated thiamine, by use of microbiological methods, a pyrimidine and a thiazole moiety (2-methyl-4-amino-5-hydroxymethylpyrimidine and 4-methyl-5-hydroxyethylthiazole) which could have arisen from the rupture of the thiamine molecule by the addition of water. They also indicated the formation of an intermediate compound which was then transformed to the above pyrimidine compound. Barnhurst & Hennessy (495) have developed a method of assay for this intermediate compound "icthiamine" and a method of isolation which has led to preparation of the compound ($C_8H_{14}N_4O_3S$) from thiamine inactivated by clam tissue. Ultraviolet absorption spectra, potentiometric titration, and reactions with Raney nickel and sodium bisulfite, together with the isolation of reaction products, indicate that there is present in "icthiamine" a 2-methyl-4-aminopyrimidine-5-methylene moiety. "Icthiamine" after treatment with bisulfite, is not resynthesized to thiamine by yeast in the presence of the thiazole moiety, while 2-methyl-4-amino-5-hydroxymethylpyrimidine will undergo such a resynthesis after bisulfite treatment. These contrasting reactions form the basis of the assay method for "icthiamine." Fujita *et al.* (496) have extended the previous observations that certain aromatic amines stimulate the reaction of thiaminase, by showing that the thiaminase obtained from shellfish, fish, and certain bacteria is stimulated by many primary aromatic amines. Secondary aromatic amines, aliphatic amines, and compounds with the amino group not directly attached to the ring, have no effect. Pyridine and quinoline are active in this respect and form compounds in the reaction mixture in which they replace the thiazole moiety of the thiamine molecule [Fujita *et al.* (497)]. This is further evidence that the breakdown of thiamine by thiaminase is an exchange reaction. The thiaminase preparations of Fujita *et al.* (497) were inactivated by dialysis, or by precipitation with acetone. The activity was re-established by the addition of the thermostable extracts or dialysates, or by addition of aromatic amines. It seems likely that the natural coenzyme for this reaction is also an aromatic amine [Fujita & Tashiro (498)]. If so, the intermediate formed must readily undergo further reaction, since both the pyrimidine and thiazole moieties are found as products of the reaction. The reversibility of the reaction is indicated by the synthesis of thiamine from its pyrimidine and thiazole moieties. However, the equilibrium point of the reaction lies much in favor of the inactivation [Fujita *et al.* (499)]. The differences noted in thiaminase preparations from different sources seem attributable to slight differences in the apoenzymes, not to a basic difference in the mechanism of reaction.

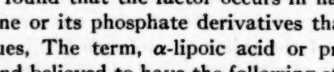
Miscellaneous studies.—Matsukawa & Iwatsu (500) have shown by synthesis and Karrer & Krishna (501) by reduction to dihydrothiamine that the product isolated after heating thiamine disulfide in ethylene glycol is N-[2′-

methyl-4'-amine-pyrimidyl-(5')]-methyl-4-methyl-5-β-hydroxyethyl-thiazolone, a probable intermediate compound in the formation of thiochrome from thiamine by alkaline ferricyanide oxidation.

Phillips *et al.* (502) have studied nine patients with Wernicke's syndrome, showing ophthalmoplegia, nystagmus, ataxia, and mental disturbance. The patients were maintained on purified diets and the effect of the addition of specific vitamins studied after appropriate intervals of observation. Prior to administration of thiamine no improvement was noted upon the addition of any of the other available vitamins. When thiamine was administered there was a prompt and considerable improvement in the ophthalmoplegia and a gradual diminution in nystagmus and ataxia in some of the patients. The improvement in mental disturbance was minimal.

Lipoic Acid (Thioctic Acid, Protogen, Pyruvate Oxidation Factor, Acetate Formation Factor)

As predicted by Snell & Wright (406) in the 1950 review, the factor required for growth of *Tetrahymena geleii* W, the factor that replaces acetate for growth of *L. casei* on an acetate free medium, and the factor that is required by *S. faecalis* for oxidation of pyruvate, have been found to be identical. The apparently pure crystalline compound has been prepared by two groups of investigators and its biological activity established [Reed *et al.* (503); Patterson *et al.* (504)]. Most details of the structure have been worked out [Reed *et al.* (505, 506); Brockman *et al.* (507)] and synthesis of the compound has been reported by two groups of investigators [Bullock *et al.* (508, 509); Hornberger *et al.* (510)]. Reed *et al.* (511 to 514) and Gunsalus *et al.* (515) have found that the factor occurs in natural materials conjugated with thiamine or its phosphate derivatives that, in turn, occur in bound form in tissues, The term, α-lipoic acid or protogen-A, has been given to the compound believed to have the following structure:



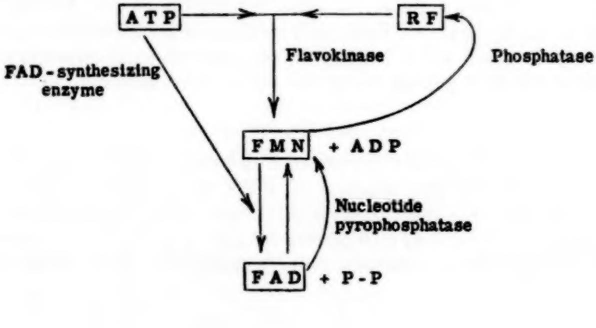
The term, β-lipoic acid or protogen-B, has been given to a form of the compound (thought to be a sulfoxide) that appears as a result of oxidation during isolation of the disulfide form of the compound, or by treatment with hydrogen peroxide. Reducing agents, e.g., hydriodic acid, convert the sulfoxide form (?) to the disulfide form. Saponification with excess sodium hydroxide produces a form of the compound containing one —SH group and reduction with sodium borohydride produces a form containing two —SH groups. There is evidence that all of these various forms are biologically active. Octanoic acid is produced on treatment with Raney nickel. α-Lipoic acid is soluble in organic solvents, but only sparingly soluble in water (m.p. 47.5–48.5°C., pK 4.7, M 220 to 230). Although there is some evidence that the secondary sulfur in the natural product is attached to carbon-five, synthetic

compounds in which the attachment is at carbon-four, -five, or -six indicate that the 6, 8-compound has by far the greatest biological activity and an activity equivalent to that of the natural compound. The term, thioctic acid, has been given to this series of compounds by one group of investigators, and it is suggested that the numerical prefix indicate the position of the carbon to which the secondary sulfur is attached, i.e., 6-thioctic acid, etc. It may be that isomers occur, based on the position of the secondary sulfur. In addition, each of such isomers could exist in a D or L form. Reed *et al.* (511, 514), in a series of beautiful experiments, have shown by the use of chromatographic and bioautographic techniques that there exist in Difco yeast extract and in liver extracts prepared by enzymatic or acid hydrolysis, complex forms of α-lipoic acid that support the growth of *Streptococcus lactis* in the absence of thiamine in the basal media, whereas, the presence of thiamine is necessary for a growth response upon the addition of α-lipoic acid. A mutant strain of *E. coli* has been obtained that requires such a complex form for growth, and it has been shown that wild-type *S. lactis* and *E. coli* produce this complex form when incubated with thiamine and α-lipoic acid, a function which the mutant has lost because of the absence of the enzyme system which synthesizes the complex. These observations, plus the fact that the conjugate gave a negative thiochrome test and a positive azo test, suggested that the conjugate contained α-lipoic acid and thiamine conjugated through an amide linkage. The preparation of synthetic compounds has proven the hypothesis that α-lipoic acid exists in tissues as lipothiamide or rather, probably, as the pyrophosphate of this amide. Lipothiamide may occur in the hydrolysis products of tissues either in the free form or as a phosphate derivative. In turn, this is bound to protein to produce the active enzyme systems that are necessary for the oxidation of pyruvate and α-ketoglutarate. The growth requirements of the mutant *coli* for lipothiamide can be completely bypassed by supplying the products (acetate, citrate, and succinate) of these two blocked reactions. The details of the mechanism by which α-lipoic acid converts pyruvate to acetate and α-ketoglutarate to succinate are not yet clear. Barner & O'Kane (317) have shown that *S. faecalis* can oxidize acetaldehyde and ethanol to acetate in the absence of pyruvate oxidation factor, and from this and what is known of other pathways of pyruvate metabolism, suggest a possible role for this factor. Although there is as yet no evidence that α-lipoic acid conjugases are involved in fat oxidation, the speculations of Lardy (322) on the possible role of enzymes containing reduced disulfide groups are of interest in this connection.

Riboflavin

Dimant *et al.* (516) have developed a procedure, based upon chromatographic methods, for the isolation of FAD (flavin-adenine dinucleotide) from animal tissue and yeast. FAD is obtained in 40 to 50 per cent yield with a dry weight purity of 0.40 to 0.60; the remaining impurity is innocuous in various enzyme systems. Methods for establishing the purity of the product

are considered in detail. An unidentified compound (FAD-X) that appears to be a new dinucleotide containing riboflavin was obtained as a result of the chromatography. Christie *et al.* (517) have synthesized a compound that is identical with FAD from natural sources in chromatographic behavior, chemical properties, and absorption spectra, and when combined with the apoenzyme of D-amino acid oxidase gives a fully active reconstituted enzyme. This synthesis not only provides a source for the compound, but, in addition, establishes the correctness of the structure and provides a method of synthesis for several other nucleotide coenzymes. Lowe & Clark (518) have determined the E_h-pH relationships of purified FAD throughout the pH range of 2 to 12. The 50 per cent reduction potentials approximate and parallel those previously reported for riboflavin. Attempts to determine the free energy of association of the reduced and oxidized forms of FAD for the apoenzyme, D-amino-acid oxidase, failed, since the deaerated apoenzyme was capable of oxidizing reduced FAD ($FAD \cdot H_2 + R—S—S—R \rightarrow FAD + 2RSH$). Synthesis of riboflavin-5′-phosphoric acid, which shows "coenzyme" activity equivalent to that of the natural riboflavin phosphate, has been described by Viscontini *et al.* (519). Whitby (520) has found by use of chromatographic methods that the homogenates of rat liver and aqueous extracts of the acetone powder produce a riboflavinyl glucoside from added riboflavin, which is probably 5′-D-riboflavin-D-glucopyranoside. Preliminary studies of this reaction indicate that an enzyme catalyzes a transglucosidation of D-glucose from maltose or glycogen to riboflavin. The compound has not been detected as a natural product in tissue or in urine, and no biological function has been suggested. That the transglucosidation may be nonspecific has not been excluded. The partial purification and the studies by Kearney *et al.* (520a, 521, 522) and Englard (523) of enzymes from autolyzed yeast and rat intestine (flavokinase) that catalyze the essentially irreversible reaction, RF (riboflavin) + ATP[2] → FMN (flavin mononucleotide) + ADP,[2] and the studies by Schrecker & Kornberg (524) of enzyme preparations from yeast (FAD-synthesizing enzyme) that catalyze the reversible reaction, $FMN + ATP \rightleftarrows FAD + $ inorganic pyrophosphate, make it possible to outline the main features of riboflavin metabolism in yeast. Kearney & Englard (521) have summarized these as follows:



The flavokinase-catalyzed reaction is stoichiometric, follows zero order kinetics, is linear with enzyme concentration, and requires the presence of a divalent cation (Zn^{++}, Mg^{++}, Co^{++}, or Mn^{++}). The dissociation constants of the enzyme for riboflavin, ATP, ADP, and AMP (adenosinemonophosphate) have been determined. ADP is about half as effective as ATP as a phosphate donor with the yeast preparation, but inactive with the rat intestine preparation. AMP is a competitive inhibitor. The optimum pH is 7.8 to 8.5. Arabitylflavin and dichloroflavin are phosphorylated by the systems while isoriboflavin, galactoflavin, dulcitylflavin, and sorbitylflavin are not. Excess lumiflavin inhibits the reaction. The FAD-synthesizing enzyme requires Mg^{++}. ATP is not replaceable by ADP or AMP. In the reverse reaction, inorganic pyrophosphate is not replaceable by ortho- or metaphosphate. Both preparations still contain small amounts of other enzymes.

Apparently, diethyl riboflavin is an adequate substitute for riboflavin in some biological systems while in others it is an inhibitor. *L. casei* has been grown for six serial generations with the diethyl analogue serving as the sole source of flavins [Lambooy (525)] while rats receiving this substitute failed to survive unless enough riboflavin is given to overcome its inhibitory effects [Lambooy & Aposhian (526)]. However, that the analogue can substitute for riboflavin in some systems in the rat is indicated by the temporary growth response resulting from its administration. As would be expected, the growth of Walker rat carcinoma is considerably inhibited by diethyl riboflavin [Aposhian & Lambooy (527)]. Holly *et al.* (528) have prepared four riboflavin analogues, 9-(1′-glycityl)-isoalloxazines, that have been found effective in enhancing the rate of regression of established lymphosarcoma implanted in mice. The riboflavin analogue, 6-chloro-9-(1-D-sorbityl)-isoalloxazine, has been found to have no effect on the growth of transplantable mammary carcinoma. However, the combination of this analogue and 8-azaguanine was carcinostatic only in female mice. Riboflavin phosphate reversed the carcinostasis [Shapiro & Fugmann (529)].

Suvarnakich *et al.* (530) and Guzman & Scrimshaw (531) have reported blood serum analyses on 141 well nourished normal adults in the United States and on 275 school children in Guatemala respectively. The values are of the same order of magnitude as those previously reported by Burch *et al.* (532), i.e., 1.5 to 5.0 μg. per cent, and show considerable scattering. Although animal studies [Brown (533); Mann *et al.* (534)] would seem to indicate that there is a predictable relationship between dietary intake and blood plasma level of riboflavin, it is evident from the great variation found among individuals maintained on constant intakes that factors other than the diet influence plasma riboflavin content greatly [Bessey *et al.* (535)]. Riboflavin deficiency in the baby pig [Lehrer & Wiese (536)] and in the Cebus monkey [Mann *et al.* (534)] has been described. According to Miller & Ellis (537) 0.83 mg. riboflavin per pound of diet was found adequate for growth for the baby pig while 0.53 mg. was inadequate. Draper & Johnson (538) have found that 1.05 μg. of riboflavin per gram dry weight of diet is optimum for the Holstein calf. Brisson & Sutton (539) have reported data indicating that

the daily riboflavin requirements just adequate to prevent deficiency symptoms (minimum requirements) in the eight-week old male calf are 35 to 45 μg. per kilogram of body weight. The direct relationship between nitrogen and riboflavin retention, which has been observed repeatedly in animals [Doisy & Westerfeld (540)] when kept on levels of riboflavin that are adequate, but not excessive, has been confirmed by Pollack & Bookman (541) with a number of human subjects. This is a factor that must be considered in using urinary excretion as a measure of dietary intake of riboflavin. Arnold *et al.* (542) have shown that riboflavin solubilized in 3-hydroxy-2-naphthoate is stable and biologically available to chick and *L. casei*. That riboflavin may be less available in the pea, soybean, and similar foods than in milk, because of incomplete digestibility, is indicated by the work of Everson *et al.* (545). Rancid fat seems to increase the requirements for various essential factors. This is true also for riboflavin, as indicated by the work of Kaunitz *et al.* (546) in which the accentuation of signs of riboflavin deficiency was observed when 10 per cent rancid fat was added to the diet of the rat. Tables of riboflavin content of some Brazilian foods [Cramer & Da Conceição (543)] and foods of India [Ghosh & Rajagopal (544)] will be found helpful in those countries. Lactose has been found by De & Roy (547) to provide a more favorable medium for the biosynthesis of riboflavin in the gastrointestinal tract of the rat as compared with diets containing starch or sucrose. Similar observations have been reported previously with respect to biosynthesis of several other nutritive factors. Xanthine, adenine, guanine, uric acid, and possibly hypoxanthine have been found by MacLaren (548) to be effective in promoting riboflavin production as much as 40 per cent, in a manner independent of the growth of the fungus *Eremothecium ashbyii* when cultured in a synthetic medium. It is suggested that one or more of these substances is incorporated directly into the riboflavin molecule. Tracer studies would seem in order in the problem. Axelrod *et al.* (549) have studied the carbohydrate metabolism in riboflavin deficiency in the dog by measurement of glucose tolerance, blood sugar, glycogen, hemoglobin, blood cell chlorides, serum protein, and nonprotein nitrogen. Impaired adjustment to cold stress has been demonstrated in a number of the vitamin deficiencies. Ershoff (550) shows this is also true for riboflavin deficiency in the rat. Stich (551) has reported the maintenance of two cases of congenital porphyria symptom free and at a low level of porphyrin excretion for 6 months and 18 months respectively, by administration of 10 mg. of riboflavin daily. If confirmed with more cases, this is an important observation.

Miscellaneous

Lyxoflavin.—In 1949, Pallares & Garza (552) reported the isolation of a flavin from human myocardium that, on the basis of comparison of composition and melting point with a synthetic product and its tetraacetate they concluded was L-lyxoflavin. As a result of the similarity in structure to riboflavin and the 1-α-D-ribofuranoside-5-6-methylbenzimidazole moiety present

in the cobalamins (vitamin B_{12}), Brink *et al.* (553) and Emerson & Folkers (554) suggested this compound might have vitamin properties. While subsequent studies with synthetic L-lyxoflavin [Emerson & Folkers (554); Heyl *et al.* (555)] give some support to this idea, a biological function for this compound has not been clearly established, and the evidence for its identification and occurrence in nature must be considered meager until additional evidence is forthcoming. Emerson & Folkers (554) report significant growth response to 150 μg. of synthetic lyxoflavin daily in rats receiving a basal diet (soybean, dextrose, Crisco, salts, known vitamins, and 0.5 per cent thyroid powder) designed for tests for the presence of new vitamins in various materials. A 15-day test period, following a period of 28 days after weaning on the basal diet only, gave a growth response comparable to the addition of 10 per cent water insoluble liver solids. Wahlstrom & Johnson (556, 557) report that the addition of 4 μg. of synthetic L-lyxoflavin per gram of solids to a low fat basal ration containing 0.01 per cent protamone led to an increased gain of weight and efficiency of feed utilization in baby pigs. The number of animals used and the benefits derived, while apparently significant, are not large. Experiments by Bruins *et al.* (558) with chicks kept on a soybean-sucrose diet supplemented with the known vitamins also showed a small growth response to L-lyxoflavin (3 mg./kg. diet). The response was of the same order of magnitude as that given by 4 per cent liver residue or 5 per cent wheat bran, but not so much as that given by 4 per cent dried whey. Ershoff (559) has found lyxoflavin (100 mg./kg. diet) ineffective compared with liver residues in prolonging the survival time and counteracting the retardation in body and gonadal weight in thyroid fed rats on a casein-sucrose diet supplemented with the known vitamins. The effects cited above were supposedly not attributable to a riboflavin substitution effect, since this vitamin was included in the basal diets. Cooperman *et al.* (560) have obtained small growth responses to lyxoflavin by *L. casei*, the rat, and the chick, but believe these are a result of a limited capacity of the compound to replace riboflavin. Lyxoflavin is not toxic for the mouse at levels 2,000 times the requirement of this species for riboflavin. Preliminary tests on patients (three) reveal no evidence of change or toxicity. That the small responses reported above could be caused by the nonspecific effects of lyxoflavin on intestinal condition, such as is the case with certain antibiotics and other nonessential substances, is a possibility that can not be dismissed at present.

Vitamin B_T.—Carter *et al.* (561) have identified vitamin B_T (meal worm factor) as carnitine by comparison of a number of properties of the factor isolated from liver and whey with carnitine isolated from commercial beef extract and a synthetic specimen. The isolated factor is identical with carnitine with respect to empirical formula, specific rotation, melting point, quantities present in beef extract, biological activity, and melting points of a variety of derivatives. Assays suggest that synthetic DL-carnitine is about half as active as the naturally occurring (—)-isomer. β-Hydroxy-γ-aminobutyric acid, which differs from carnitine [$(CH_3)_3N^+$—$CH_2CH(OH)$

$CH_2CO_2^-$] only by lack of methylation of the amino group, shows vitamin B_T activity at a level of 12 to 24 μg./gm. of diet, compared with 0.37 to 0.75 μg./gm. of diet shown by carnitine. Apparently, carnitine can be formed to some degree by methylation of the corresponding amino acid. Vitamin B_T is most conveniently prepared from beef extracts which contain 1.5 to 3.0 per cent carnitine. Whey, yeast, and liver are also good sources. Most animal tissues show good activity. Eggs and most vegetable materials, except wheat germ, are poor sources [Fraenkel (562)]. The factor was discovered and named vitamin B_T in 1948 by Fraenkel *et al.* (563, 564) when attempts to grow the meal worm *Tenebrio molitor* indicated that in addition to known B-vitamins, a factor present in charcoal eluates from yeast and liver extracts was required for normal growth and survival of the *Tenebrio* larvae beyond the fourth week of development. The factor was isolated by phenol extraction of aqueous extracts of liver and whey [Fraenkel (565)], alumina chromatography, and counter current distribution in a phenol-dilute hydrochloric acid (pH 1.5) system and crystallization from acetone-ethanol. The free vitamin was obtained from the hydrochloride with an anion exchange resin and crystallized from anhydrous acetone-ethanol (561). Carnitine is highly hygroscopic, stable in acid, slowly inactivated by strong bases, not easily affected by ultraviolet light, and adsorbed on fuller's earth. It may be autoclaved at 125°C. for three hours at pH 2.5 and 7.7 without loss of activity. Carnitine is required also by *Palorus ratzeburgi*, a meal worm belonging to the same family as *Tenebrio*. Tests by collaborating workers have noted no effects on the chick, rat, guinea pig, *Tetrahymena*, and several species of bacteria (561).

Biotin.—Biotin has been implicated in numerous metabolic processes including (*a*) the carboyxlation and decarboxylation of acetoacetate, oxalacetate, aspartate, and malate; (*b*) the deamination of aspartate, serine, and threonine; and (*c*) the synthesis of citrulline and unsaturated fatty acids [Lichstein (566)]. The recent preparation of the "coenzyme" of aspartic acid deaminase (*Bacterium cadaveris*) by sulfuric acid degradation of carbohydrate indicated that biotin is not directly related to this reaction [Christman & Williams (567)]. The material was identical in its coenzymatic and physico-chemical properties with the coenzyme in yeast extract. The criteria of restored ammonia production in aged or phosphate washed bacteria may not in itself be sufficient to establish a factor as coenzyme for the aspartic acid deaminase reaction. Williams & Williams (568) have suggested that biotin is involved in only one primary system whose failure manifests itself in the secondary metabolic derangements. The effect of biotin in eliminating the diffusion current maximum encountered during the polarographic reduction of cupric ions has been confirmed by Wright & Shultz (569). Biocytin showed similar "surface active" effects equal to those expected from its biotin content. The isolation of several milligrams of crystalline biocytin from 25 tons of yeast has been carried out by Wright *et al.* (570). The compound was identified as ε-N-biotinyl-L-lysine [Wright *et al.* (571); Peck *et al.*

(572)] and the structure confirmed by synthesis from biotinyl chloride and L-lysine [Wolf *et al.* (573)]. All biological comparisons confirmed the identity of isolated and synthetic biocytin [Wright *et al.* (574)]. The impairment of growth of *Mycobacterium tuberculosis* by 4-(imidazolidone-2)-caproic acid, homobiotin, and norbiotin was reversed by biotin. Desthiobiotin and oxybiotin functioned as metabolites for the bacilli [Pope (575)]. In the rat, the intraperitoneal injection of 0.01 to 0.02 mg. of biotin resulted in urinary excretion of 40 per cent of the dose. Labeled C^{14}-biotin was similarly excreted within one day, in a microbiologically inactive form. When administered as the radioactive biotin-avidin complex, 50 per cent was excreted in three to five days as free biotin. Seven per cent of the radioactive dose was in the liver three to six days after administration, largely as biotin [Fraenkel-Conrat & Fraenkel-Conrat (576)]. Lewis & Everson (577) have obtained information on the daily transfer of pantothenic and biotinic acids from the pregnant stock to the developing fetuses. The highest requirement for biotin occurred around the twentieth day when the rate of deposition of biotin exceeded 3 μg. per 24 hours. The synthesis of DL-*epi*-biotin, DL-*epi-allo*-biotin from ω-carbomethoxyvaleraldehyde, nitromethane, mercaptoacetaldehyde-acetal, and ammonia has been described [Grob & von Sprecher (578)]. The relation of vitamins (including biotin) to antibody production in rats immunized to human erythrocytes has been investigated by Axelrod (338).

Inositol.—The biochemistry and determination of the cyclitols, inositol, phosphate esters of inositol, and *meso*-inositol in microorganisms, plants and animals, have been reviewed in a recent symposium [Posternak (579); Courtois (580); Fleury (581); Schopfer (582)].

The urinary excretion of inositol in a number of animals has been reported by Fleury *et al.* (583). Courtois *et al.* (584) have noted an inhibition of phytase hydrolysis of inositol phosphates in the presence of aluminum and iron salts. Mann (585) reports that 40 per cent of the dialyzable dry matter of boar seminal vesicle secretion is inositol.

Determinations by Volk & Pennington (586) suggest that *Propionibacterium pentosaceum* effects a dismutation of inositol to hexahydroxybenzene, propionic acid, acetic acid, and carbon dioxide. The hexahydroxybenzene was autoxidized to tetrahydroxyquinone and rhodizonic acid. Glucose could not be identified as an intermediate in inositol fermentation. The oxidation of inositol by *M. tuberculosis avium* was accelerated by addition of phosphate. The presence of asparagine and inositol resulted in accelerated growth of this organism [Nagaya (588)]. The preparation of a cell free enzyme from *Acetobacter suboxydans*, capable of oxidizing *meso-d-* and *epi*-inositol, *d*-quercitol, and *dl-epi*-inosose has been described by Franzl & Chargaff (589). The heat labile enzyme preparation was inhibited by potassium cyanide and colchicine (noncompetitive type). The rate of growth of *Saccharomyces cerevisiae* in an inositol free medium is determined by the limiting rate of biosynthesis of inositol [Pennington *et al.* (590)]. Inositol removed from the media by growing *Nematospora gossypii* and *Saccharomyces carlsbergensis*

was recovered in part by water extraction and in 75 per cent yield by acid hydrolysis of the organisms. This bound inositol from yeast resembled phytin. γ-Hexachlorocyclohexane irreversibly inhibited growth and respiration of intact yeast cells. Streptidine, strepturea, and streptamine had no effect in replacing inositol or in inhibiting respiration of yeast [Smith (591)]. Srivastava (592) has noted that roaches bred and reared on *meso*-inositol show increased resistance to γ-hexachlorocyclohexane. Inositol inhibited the expected rise in cholesterol and phospholipid levels in serum of rabbits on a high cholesterol diet [Dotti *et al.* (593)]. The administration of 2 to 6 gm. doses of inositol daily failed to alter the occurrence of atherosclerosis in male albino rabbits fed from 0.5 to 2.5 gm. of cholesterol daily [Moses *et al.* (595)]. Thirty-seven patients with arteriosclerosis obliterans, taking 1 gm. of inositol daily, showed no improvement after 12 to 18 months of therapy [Popkin (594)].

Choline.—The relationship of choline to the various aspects of fat metabolism were reviewed last year by Frazer (596). The present review is concerned primarily with researches on the highly important lipotropic action of choline and methyl groups. The role of vitamin B_{12} in the metabolism of choline is discussed in the review of the vitamin.

Rats on high cholesterol hypolipotropic diets customarily show an accumulation of liver lipids (cholesterol and glycerides) and hypercholesterolemia. Under these conditions, Ridout *et al.* (597) have observed that choline is capable of maintaining liver glycerides near normal while cholesterol levels in the liver remain slightly elevated. Inositol showed negligible lipotropic action. The lipid content of several human diets was reported. Weiss *et al.* (598) have noted that under similar conditions thyroid administration diminishes both liver lipids and hypercholesterolemia. (Microscopic crystals of cholesterol were observed in the aortic endothelium of the thyroid treated rats.) Choline plus inositol exerted its customary lipotropic action on liver lipids, did not affect the aorta, and unexpectedly accentuated the hypercholesterolemia. Rats on low protein (nitrogen) diets develop nutritional edema, anemia, fatty metamorphosis, and cirrhosis of the liver. Alexander & Engel (599) have stated that choline is capable of preventing or correcting the edema and anemia. Plough *et al.* (600) have noted, however, that the production of liver cirrhosis in rats on a 4 per cent casein diet is not arrested by addition of choline or methionine to the diet. A diet of 30 per cent casein (with or without added choline) resulted in regression of the pathology. The therapeutic value of high protein diets was stressed. Employing P^{32}, Campbell & Kosterlitz (601) have reported that in the adult rat the liver content and turnover of desoxyribonucleic acid phosphorus is constant and unaffected by dietary protein, fat, or choline. The phospholipid phosphorus content of liver was determined mainly by protein intake and not at all by the choline content of the diet. Desoxyribonucleic acid phosphorus was suggested as a standard of reference for other liver constituents. Rose *et al.* (602) have postulated an *in vivo* source of glycine, serine, cystine, or choline, since

the growth of weanling rats is not significantly affected by removal of these compounds from a diet containing minimal amounts of methionine and threonine as well as other purified essential amino acids and vitamins. Only under conditions of extreme depletion of the above named nonessential amino acids was it possible to impede growth. Interestingly enough, liver disease is the only disease thus far known to result in a more rapid absorption of choline and consequent reduced urinary excretion of trimethylamine [de la Huerga & Popper (603); Popper *et al.* (604)]. In view of these results, the experiments of Baxter & Campbell (605) are of interest. The renal lesions and animal mortality caused by a purified diet deficient in choline were largely prevented by supplementation of the diet with rather high levels of crystalline aureomycin, which increased tissue and fecal content of choline. All of these results suggest a free exchange of choline between the intestine and portal blood, and one would predict a decreased excretion of trimethylamine. The observed increase in fecal choline supports such a hypothesis. The proper interpretation undoubtedly involves both intestinal decomposition as well as vitamin B_{12}-controlled synthesis of choline. Other causes of fatty metamorphosis of the liver are of practical clinical interest. In carbon tetrachloride intoxication of rats, Hartmann *et al.* (606) have found beneficial lipotropic effects of vitamin E, methionine, inositol, and invert sugar. Methionine and choline significantly prolonged the survival time of rats receiving toxic doses of pentothal [Enders & Körner (607)]. Forsander & Hallman (608) have indicated that choline or lipocaic deficiency or both is not the cause of fatty metamorphosis of the liver in the gastroenteritis of infants. Their values for free choline in plasma (60 to 80 μg./ml.) were considerably higher than those reported for normal adults by the recent method of Bligh (609). The details of tissue inactivation, specificity of extraction, and final assay methods (chemical, microbiological, or neuromuscular) deserve the closest scrutiny. Schmidt *et al.* (610) have developed a procedure for the quantitative determination of glyceryl phosphoryl choline which may constitute one half of tissue phospholipid choline. The specific activity of liver fatty acid and liver, heart, and serum cholesterol of normal and vitamin deficient (thiamine, pantothenate, biotin, choline) rats administered radioactive carboxyl-labeled acetate were found to be identical [Guggenheim & Olson (611)]. However, the deficient animals showed altered amounts of total lipid activity. It seems clear that these deficiencies did not make rate limiting the reactions or factors catalyzing the equilibrium between acetate and lipid stores, but variously affected endogenous lipotropic agents. The capacity of dietary choline to increase the amount of respiratory C^{14}-carbon dioxide arising from methyl-labeled methionine has been reported by Mackenzie & du Vigneaud (612). Cystine restored the respired C^{14}-carbon dioxide to control levels. It is conceivable that the primary requisite of the rat under these conditions is for cystine (cystathione); the fate of the methyl groups would then depend upon the availability of methyl acceptors (low in the presence of choline).

Choline oxidase (CO) is located principally in the mitochondria. The par-

tially inactivated CO (freeze-thaw method) is reactivated by ATP.[2] Dinitrophenol has no effect on CO. Boiled extracts of CO reportedly increased the activity of frozen and thawed mitochondrial CO [Williams (613)]. Riboflavin deficiency decreases the CO level of weanling rat liver; vitamin B_6 and thiamine deficiencies have no effect [Richert & Westerfeld (614)]. The cellular distribution of vitamin B_{12} is reportedly similar to that of CO. The minimal lethal dose of choline for rats is 70 to 75 mg./kg. It has been shown that choline affords no protective action against nitrogen mustard intoxication [Howe *et al.* (615)]. Pyrophosphorylcholine has been synthesized and its hydrolysis by various phosphatases studied [Roche and co-workers (616, 617)]. It appears inactive as a cofactor of phosphatase. Growth inhibition of *S. carlsbergensis* caused by choline and dimethylaminoethanol, but not by ethanolamine, serine, betaine, methionine, or choline phospholipids has been reported by Taylor & McKibbin (618).

Miscellaneous factors.—A number of reports have appeared that strengthen the view that there exists in blood hitherto unidentified factors capable of promoting optimal growth of a variety of organisms. Positive identification of any of these factors has not been made so that their relation to one another remains unknown. Steinman *et al.* (619) have described a medium suitable for cultivation of Reiter treponeme, which contains amino acids, vitamins, purine, pyrimidine, carbohydrate, inorganic ions, and crystallized serum albumin. When *Trypanosoma cruzi* was subcultured from a blood coagulum medium into a series of media containing graded concentrations of thrice recrystallized hemoglobin, growth was found to be proportional to the amount of hemoglobin present. Hemin, acid or peptic hydrolysates of hemoglobin, and a heme-globin mixture could not be substituted for hemoglobin. Ascorbic acid or serum added to unheated hemoglobin gave positive growth responses through six to eight subcultures respectively [McRary *et al.* (620)]. A satisfactory nutrient medium for *Staphylococcus albus* was devised for the assay of a growth stimulatory factor SSF (cattle blood plasma factor). Of the natural materials assayed, the human blood plasm fraction IV-A was found to be most active in reducing the lag phase of growth. SSF was obtained as a polypeptide from glycoprotein of oxalated bovine plasma. Similar peptides were isolated from several recrystallized proteins, including protein from certain urine specimens [Sloane & McKee (621)]. Griffin (622) has repeatedly grown and subcultured *Hemophilus piscium* in a medium containing various amino acids, adenine, guanine, uracil, glucose, several vitamins (including cocarboxylase), and hemin. Competitive inhibition of growth by thiamine was reversed by cocarboxylase and ATP.[2] A low molecular weight protein growth factor, isolated from mammalian blood serum or ascitic fluid, was required for growth by pleuropneumonia-like organisms [Smith & Morton (623)].

Hoffman & Pavcek (624) have identified the yeast extract growth factor for *S. faecalis* as uridine. Tween 80 and Triton A-20 act synergistically with uridine to give a growth response equivalent to that obtained with yeast

extract. The effect of these surface-active compounds is reminiscent of their similar effect on the action of biotin [Williams & Williams (568); Wright & Shultz (569)] and a biotin-like factor derived by acid hydrolysis of carbohydrates [(567); (568); (569)]. The existence in yeast of natural esters of fatty acids simulating Tween 80 was suggested. On the other hand, Lichstein & Boyd (625) have demonstrated the activation of a formic dehydrogenase system (from a biotinless mutant of *E. coli* and a normal strain of *A. aerogenes*) by oleic and other long chain fatty acids. Yeast and liver contained a factor more active than oleic acid. The activity of natural material was increased by acid hydrolysis. A procedure for the assay of strepogenin activity in *L. casei* was applied to the assay of strepogenin in highly purified proteins (insulin and liver). Glutamine, asparagine, and aspartic acid showed a slight strepogenin activity; glutamic acid and glutathione were inactive. The activity of insulin was found to be related to an integral peptide part of the molecule [Kodicek & Mistry (626)]. Shantz & Steward (627) have crystallized three closely related factors from coconut milk [C.M.F. (coconut milk growth factor)] that possess the ability to incite random cell division in plant tissues. Dietrich *et al.* (628) have found evidence that an additional growth factor is required by the chick and hyperthyroid rat. Rats receiving iodinated diet grew 30 per cent better on a dextrin diet than those on a sucrose basal diet. Alcohol extracts of yeast and powdered liver, casein, and wheat bran, were 15, and 6 to 8 times as active respectively as those of dextrin. The capacity of purines to promote growth of 17 species of mold has been described by Fries (629). Sulfonamide resistant *Staphylococcus aureus* produces a diazotizable amine that has been positively identified as *p*-aminobenzoic acid [Leskowitz *et al.* (630)]. Huffman *et al.* (631) have demonstrated a possible deficiency of a lactation factor in hay crops. This factor could be replaced by grain extracts and was not related to dietary fiber content (632). Menge *et al.* (633) have observed growth responses 15 to 20 per cent above normal in chicks on a diet containing alcohol extracts of liver preparations, dried yeast, or dried whey. A seasonal difference in the requirements of the chick for an unidentified growth factor in the juice of forage crops was found to be related to the dietary intake of this growth factor by the parent hen. This emphasized the importance of the dietary history of the strain in nutritional studies [Kohler & Grahan (634); (635)].

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CHEMISTRY OF AMINO ACIDS, PEPTIDES, AND PROTEINS[1]

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I. AMINO ACIDS

New or Uncommon Naturally Occurring Amino Acids

Various chromatographic techniques, and in particular paper chromatography, continue to prove valuable methods either for the discovery of new amino acids existing in the free state in various organs and tissues, or produced by hydrolysis of certain natural peptides, or for the detection of amino acids, already recognized but still considered uncommon, in biological systems not yet studied from this point of view.

C_3.—The presence of D-alanine in a peptide extracted from *Staph. aureus*, cultured in the presence of penicillin, briefly noted by Park (1) two years ago, has been confirmed by the same author (2). β-Alanine has been found in the free state in nodules of leguminous roots (3), in fresh or dried tea leaves (4), and, after hydrolysis, in extracts of dog, pig, and beef cerebral tissue (5). The existence of α-hydroxyalanine as a constituent of ergotamine and ergosine has been definitely established by Stoll *et al.* (6, 7). Phosphoserine has been crystallized by Agren *et al.* (8) from an enzymatic hydrolysate of casein. α,β-Diaminopropionic acid, identified among the hydrolysis products of viomycin, has been obtained in pure crystalline form by Haskell *et al.* (9).

C_4.—Hunt (3) has shown the existence of α-aminobutyric acid and γ-aminobutyric acid in the free state in the root nodules of legumes. The presence of these two acids along with β-alanine evidently results from decarboxylation of one of the carboxyl groups of glutamic and aspartic acid, respectively. γ-Aminobutyric acid, as well as β-alanine, is found in the cerebral tissue of various mammals (5). γ-Aminobutyric acid has been found by Gilligan *et al.* in gastric juice (10), and by May & Thillard (11) in the sciatic nerve of the dog and in saliva (12). Low (13) has found α-aminobutyric acid in protein hydrolysates obtained from the sponge (*Spongia officinalis obliqua*). Fink *et al.* (14) have identified β-aminoisobutyric acid in the urine of rats which have ingested thymine, and particularly dihydroxythymine. This amino acid does not occur in the urine of rats on a normal diet, contrary to its presence in normal (15) or pathological (16) human urine.

C_5.—The existence of D-valine and N-methyl-L-valine in actinomycin C has been confirmed by Brockmann *et al.* (17). D-glutamic acid, long recognized as a constituent of polyglutamic peptides from the capsule of *B.*

[1] The survey of the literature pertaining to this review was concluded in November, 1952.

anthracis or secreted by the group *B. mesentericus–B. subtilis*, has been found by Park (2) in the D-alanine-containing peptide extracted from *Staph. aureus* grown in the presence of penicillin. Done & Fowden (18) have extracted γ-methyleneglutamic acid and the corresponding amide, γ-methyleneglutamine, from vegetative parts of the peanut (*Arachis hypogaea*). γ-Methyleneglutamic acid has been identified by analysis, by hydrogenation into γ-methylglutamic acid, by a study of its oxidation products, by its x-ray spectrum, etc. In the course of a Stickland reaction, under the influence of sheep rumen bacteria (19), δ-aminovaleric acid has been isolated and characterized as a product of the reduction of L-proline coupled with the oxidation of alanine. The question of whether proline exists in the D or L form in peptides from rye ergot has been definitely answered by Stoll *et al.* (20) who have shown that it is indeed L-proline which is one of the constituents of these peptides.

C_6.—In a systematic investigation of α-aminoadipic acid in various nitrogenous substances, Windsor (21) has verified the existence of this acid in the free state in maize kernels, in the aqueous extract of these kernels (corn-steep liquor), and in takadiastase. Also, α-aminoadipic acid has been shown to be a constituent of one of the soluble proteins of corn kernels which passes into the aqueous wash. α-Aminoadipic acid has been identified and isolated by starch chromatography according to the method of Moore & Stein. The acid thus obtained was shown to be identical with the synthetic acid by elementary analysis, mixed melting point determination, behavior in chromatography on starch and Dowex-50, action as a growth factor for a mutant of *Neurospora crassa* which requires α-aminoadipic acid, and by the inhibition of this growth by a specific inhibitor. The ionization constants for α-aminoadipic acid are as follows: $pK_1 = 2.14$; $pK_2 = 4.21$; $pK_3 = 9.77$; $pI = 3.18$. This acid has also been identified in urine by Boulanger & Biserte (22).

Alloisoleucine has been shown by Brockmann *et al.* (17) to be a constituent of actinomycin C. Barbier & Lederer (23) have demonstrated the presence of hydroxylysine in the phosphatide isolated from a strain of *M. phlei*. The amino acid has been characterized by its behavior in paper chromatography in several solvents, by paper electrophoresis, and by its reaction with periodic acid. It has also been isolated and crystallized as the picrate and the hydrochloride. In the phosphatide, hydroxylysine is linked by its hydroxyl group in the δ-position and by its amino group in the ϵ-position.

β,ϵ-Diaminocaproic acid, or isolysine, is a constituent of the peptides streptothricin, viomycin, and streptoline (9, 24). It has been characterized as the di(-*p*-hydroxyazobenzene-*p*-sulfonate), and as the picrate. It gives a positive reaction with hydroxamic acid but does not react with periodic acid. Its structure has been established by comparison with synthetic β,ϵ-diaminocaproic acid, the comparison resting mainly on the infrared spectra and behavior during paper chromatography (25).

Monoiodohistidine appears to be a normal constituent of mammalian

thyroglobulin. Roche *et al.* (26) have, in fact, characterized it in thyroglobulin from rats and dogs which have previously received injections of sodium iodide labeled with I^{131}.

C_7.—α,ϵ-Diaminopimelic acid, already reported by Work (27) in culture media of *C. diphtheriae*, and characterized by Asselineau *et al.* (28) as one of the constituents of a lipopolysaccharide of *M. tuberculosis*, has now been found by Gendre & Lederer (29) in 15 different strains of *M. tuberculosis*, (among which were five human, four bovine, and one avian), in proportions varying from 0.2 to 0.7 per cent by weight of the dry bacteria. This acid has also been shown by Blass *et al.* (30) to be present in an extract of *Vibrio cholereae*.

Woolley *et al.* (31) have isolated α,ϵ-Diamino β-hydroxypimelic acid from the toxin of *Pseudomonas tabaci* of which it constitutes more than 50 per cent. The identification of this new amino acid, called tabtoxinin, rests on its analysis and the following facts: (*a*) quantitative determination of CO_2 liberated when the substance is heated with ninhydrin shows that all the nitrogen of the molecule is amino nitrogen contiguous to the carboxyl group; (*b*) the substance reacts with one molecule of periodate to give an oxidation product whose chromatographic behavior is identical with that of glutamic acid; (*c*) the deamination of tabtoxinin followed by periodate oxidation and then by oxidation of the resulting products with hypobromite, gives α-hydroxyglutarolactone in appreciable quantity. The structure of this new amino acid is thus proven without any possible doubt. The behavior of tabtoxinin in chromatography on Whatman #1 paper is given in Table I. Tabtoxinin forms a di-DNP derivative, M.P. 120–122°, with an R_f value of 0.14 in *n*-butanol saturated with 5 per cent aqueous ammonia on Whatman #1 paper in ascending chromatography.

TABLE I

BEHAVIOR OF TABTOXININ IN PAPER CHROMATOGRAPHY ON WHATMAN #1

R_f	Solvent	Direction of chromatography
0.06	*n*-propanol (2):water (1)	ascending
0.10	phenol saturated with water	descending
0.21	phenol saturated with 5% NH_3	descending
0.11	phenol saturated with 0.1 *N* NCl	descending

Derivatives of tyrosine.—Several new halogen derivatives of tyrosine have been demonstrated either in proteins of marine animals or in thyroglobulin. Monoiodotyrosine has definitely been shown to be a constituent of thyroglobulin (32) and of sponge proteins (13). Monobromotyrosine is also a constituent of gorgonians (13, 33). Diiodotyrosine and dibromotyrosine have been found by Low (13) in proteins from sponge. Diiodothyronine

(26) and triiodothyronine (34) have been found in rat thyroglobulin by a technique analogous to that used for monoiodohistidine. Thyroxin occurs also in gorgonians (33).

SYNTHESES OF AMINO ACIDS AND OF SOME OF THEIR DERIVATIVES

General reviews concerning the principal methods of amino acid synthesis have been published by Velluz (35) and Atkinson *et al.* (36). Various synthetic methods, either new, or resulting from modifications of methods already known, have been described, destined mainly for the formation of amino acids labeled in certain positions with stable or radioactive isotopes. Also new syntheses of amino acid derivatives, interesting from a biochemical viewpoint, have been described. Miller & Waelsch (37) have published a method for preparing benzyl esters of various amino acids: phenylalanine, leucine, etc., by the action of benzyl alcohol on the corresponding benzene sulfonyl derivatives. This method eliminates racemization. A whole series of N-thiolacyl derivatives of amino acids has been prepared by Sheehan & Johnson (38).

C_2.—Gluud & Klempt (39) have proposed a synthesis of glycine starting with products derived exclusively from coal (formaldehyde, hydrocyanic acid, and ammonia.)

C_3.—Synthesis of DL-alanines, each labeled on a different carbon atom, have been carried out by Ostwald *et al.* (40) by the following reactions:

$$CO_2 \rightarrow CH_3I;\ CH_3I + CO_2 \rightarrow CH_3COOH \rightarrow CH_3CH_2OH \rightarrow CH_3CH_2Br;$$
$$CH_3CH_2Br + CO_2 \rightarrow CH_3CH_2COOH \rightarrow CH_3CH(NH_2)COOH,$$

where introduction of C^{14} as $C^{14}O_2$ is made in one or another of the steps. Gawron & Lieb (41) have synthesized β-isopropyl-mercapto-L-alanine and various derivatives. Several derivatives of β-alanine, for example the ethyl ester of N-phthalloyl-β-alanine, have been described by Balenovic *et al.* (42). Crawhall & Elliott (43) have described a synthesis of cystine through the intermediary of either 2-phenyl-2-thiazoline-4-carboxylic acid or 2-benzylthio-4,4-dicarbethoxy-2-thiazoline. Balenovic & Fles (44) have prepared S-benzyl-N-phthalloyl-L-cysteine. Bourland & Pacht (45) have synthesized O-phosphorylserine by phosphorylation of serine with phenyl phosphodichloride. S-phosphoryl-cysteine has been prepared by Binkley (46) by phosphorylation of cysteine with phosphorus oxychloride in a cold alkaline medium; by acidification at pH 3 the phosphoryl radical attached to nitrogen is hydrolyzed and the resultant compound is the S-phosphoryl derivative for which various properties are given. Cysteic acid amide has been obtained by Mueller *et al.* (47) by treating N,N′-dicarbobenzoxycystinyl chloride with sodium in liquid ammonia.

C_4.—L-threonine-4-C^{14} containing N^{15} has been prepared by Meltzer & Sprinson (48) by a modification of the method of Pfister *et al.* (49); the DL derivative obtained was resolved through the N-*p*-nitrobenzoyl threonine according to Zambito *et al.* (50). Shulgin *et al.* (51) have synthesized DL-

threonine and DL-allothreonine, labelled with N^{15}, starting from α-bromo-β-methoxybutyric acid, or, labeled with C^{14} in positions 1 and 2, starting from crotonic acid containing C^{14}, and have indicated the possibility of separating threonine from allothreonine by chromatography on Dowex 50. Sprinson *et al.* (52) have also synthesized L-threonine-2-C^{14} using a modification of the method of Elliott (53). Izumiya (54) has synthesized N-methyl-L- and D-threonines. Wang *et al.* (55) have obtained DL-aspartic acid-4-C^{14} by a reaction between ethyl formylaminomalonate and methyl bromoacetate-1-C^{14}. King & Kidd (56) have prepared DL- and L-asparagine starting from the DL- and L-phthalloylaspartic acid anhydrides. Two new syntheses have been suggested for α,γ-diaminobutyric acid: one proposed by Atkinson & Poppelsdorf (57) employs β-phthalimidopropaldehyde which then reacts with HCN; the other, proposed by Rothchild & Fields (58) is an application of a general method of preparation of diamino acids employed both for the synthesis of ornithine and of lysine. This method utilizes the action of hydrazoic acid, HN_3, on a tricarboxylic acid in the presence of a strong mineral acid.

Even though they are not among the naturally occurring amino acids, it is interesting to mention the synthesis of β-aminobutyric acid starting with L-alanine (59) and that of β-amino-γ-hydroxybutyric acid by reduction of the α-benzyl ester of ethyl N-carbobenzoxyaspartyl-β-glycinate with lithium aluminum hydride (60).

Several new syntheses have been described for methionine: that of Bischoff (61) based on the reaction of α-aminobutyrolactone with the sodium salt of methyl mercaptan; that of Gresham *et al.* (62) utilizing the action of methyl mercaptan on acrolein; that of Litvak *et al.* (63) which also employs the action of methyl mercaptan on acrolein in the presence of acetyl peroxide; and that of Holland & Nayler (64), starting from β-methylthiopropaldehyde. Wood & Mills (65) have published a method of biological synthesis by yeasts, of L-methionine and L-cystine labeled with S^{35}. Methionine sulfoxenine has been synthesized by Bentley *et al.* (66) from hydrazoic acid and methionine sulfoxide. L-β-amino-S-benzyl-homocysteine has been synthesized by Balenovic & Fles (44) and S-*n*-butyl-homocysteine (butionine) by Bergmann (67). Gaudry *et al.* (68) have synthesized the higher homologues of cystine and methionine: pentocystine, pentomethionine, hexocystine, and hexomethionine.

C_5.—DL-glutamic acid 5-C^{14} has been obtained by Speer *et al.* (69) utilizing a modification of the method of Marvel & Stoddard. DL-glutamic acid-1,2-C^{14} has been synthesized by the same authors by a modification of the method of Kögl *et al.* Waller & Paterson (70) have described the preparation of a series of esters (methyl, ethyl, propyl, butyl, and benzyl) in the γ-position of glutamic acid. Touster & Carter (71) have synthesized β,γ-dihydroxyglutamic acid with 3,5-dicarbethoxy-4-hydroxyisoxazole as the starting product. Jollès & Fromageot (72) have obtained γ-amino-δ-hydroxyvaleric acid by reduction of the diester of γ-glutamylglycine with lithium

aluminium hydride. Proline has been synthesized by Sorm & Smrt (73), starting with tetrahydrofuran which is opened to give α-bromo δ-chlorovaleric acid; this is finally cyclized with ammonia to give DL-proline. The same method can be used for the synthesis of hygric acid. A method of formation of proline which obviously cannot be considered as a preparatory method, but which is interesting from an analytical point of view, is that described by Hamilton (74): proline is formed in small quantity from ornithine, citrulline, or arginine when these acids are heated with nitrous acid in the presence of 6 *N* HCl.

Allohydroxy-D-proline has been obtained by Robinson & Greenstein (75) in pure form, starting from hydroxy-L-proline; the latter, by treatment with acetic anhydride in acetic acid gives the two acetyl epimers which, after hydrolysis, are separated by crystallization from ethanol at 90°. Phospho-oxyproline has been obtained by phosphorylation of oxyproline with phenylphosphodichloride (45). Fox & Minard (76) have prepared and studied a series of new derivatives of valine, among which are: maleyl-DL-valine, maleyl-D-valine, and maleyl-L-valine; fumaryl-di-DL-valine; the ethyl ester of itaconyl-DL-valine, and itaconyl-DL-valine. β-Hydroxyvaline, not yet found in nature, has been synthesized by Tatsuoka *et al.* (77) and by Rüfenacht (78). Sugino *et al.* (79) have described a procedure for the preparation of L-ornithine, with good yield, by the enzymatic hydrolysis of L-arginine; L-ornithine is extracted from the hydrolysate and purified as the picrate. N-α-methyl-L-ornithine has been obtained by Izumiya (80) using N-δ-benzoyl-L-ornithine as the starting compound. Katchalski & Spitnik (81) have prepared ornithine anhydride, used for the synthesis of ornithine and arginine polypeptides, starting with δ-carbobenzoxy-DL-ornithine methyl ester.

C_6.—The synthesis of isoleucine labeled with C^{14} in the methyl group has been accomplished by Ceresia *et al.* (82) in the following manner:

$$ROCH_2COC_2H_5 \xrightarrow{C^{14}H_3MgI}$$

$$ROCH_2C(C^{14}H_3)(OH)C_2H_5 \xrightarrow{(COOH)_2} C_2H_5CH(C^{14}H_3)(CHO)$$

$$\xrightarrow{HCN,\ NH_3} \text{Hydantoin} \xrightarrow{H_2O} CH(C_2H_5)(C^{14}H_3)\cdot CH(NH_2)\cdot COOH.$$

Rogers (83) has suggested a synthesis of lysine starting from 5(4 chloro- or bromo-butyl)-hydantoin and ammonia in the presence of an ammonium salt. Lysine has also been synthesized by Floyd *et al.* (84) utilizing the condensation of ε-aminocaproic acid with ethyl oxalate, and by Shechter & Kirk (85) using 2-cyanocyclohexanone and hydrazoic acid as starting materials. Two methods for the synthesis of δ-hydroxylysine have been suggested: one, by Touster (86), using the (cyanhydrin of γ-acetamino γ,γ-dicarbethoxybutyraldehyde, is similar to the method already described by Sheehan & Bolhofer (87); the other, described by Van Zyl *et al.* (88) employs catalytic

reduction and subsequent hydrolysis of the (γ-hydroxy-δ-nitro-*n*-butyl) acetamidomalonate ethyl ester. N-α-methyl-L-arginine has been obtained by Izumiya (80) by guanidylation of N-α-methyl-L-ornithine . Borsook *et al.* (89) have suggested a synthesis of histidine in which the carboxyl group is labeled; this synthesis employs hippuric acid, which is obtained from glycine labeled in the carboxyl position, as the starting compound. Hippuric acid, reacting with imidazolformaldehyde, gives 2-phenyl-4-(1-acetyl-imidazole-4 (or 5)-methylidene)oxazolon, which on hydrolysis becomes α-benzoylamino-β-imidazole-4 (or 5)-acrylic acid. This latter compound, on reduction by sodium amalgam, gives benzoyl histidine, and, finally, DL-histidine.

Aromatic amino acids.—Synthesis of phenylalanine, tyrosine, and O-methyltyrosine, starting from rhodanine, have been described by Gaudry & McIvor (90). The following derivatives of tyrosine were the subject of new syntheses or investigations: O-*p*-toluenesulfonyl-L-tyrosine and its N-acetyl and N-benzoyl derivatives (91), N-succinyl-L-tyrosine and N-glutaryl-tyrosine (92). Michel *et al.* (93) have synthesized L-thyroxine labeled with I^{131} either in 3,5,3′,5′, or in 3,5 positions only. Ragni & Panattoni (94) synthesized tetrabromothyronine starting from 3,5-dibromo-L-tyrosine. Tryptophan has been obtained by Weisblat & Lyttle (95) by the action of nitroacetic acid on 3-dimethylaminomethylindole. Kotake *et al.* (96) synthesized 3-hydroxykynurenine starting with 2-nitro-3-methoxybenzoic acid. 3-4-Dihydroxykynurenine has been synthesized by Butenandt & Schlossberger (97) from 2-dinitro-3,4-dimethoxyacetophenone.

Resolution of Amino Acids into their Enantiomorphs

More or less important modifications of already known methods have been utilized for the resolution of various amino acids into their optical isomers. The enzymatic method of Greenstein continues to play an important role. An attempt at a general separation of optical isomers by paper chromatography, using an asymmetric solvent, *l*-methyl (β-phenylisopropyl) amine ($[\alpha]_D = -17°8$) was made by Kotake *et al.* (98); the method did not give satisfactory results with the leucines, but achieved a partial separation of acidic amino acids (glutamic acid and tyrosine), and complete separation of tyrosine-3-sulfonic acid. Another fairly general method of separation has been proposed independently by Albertson (99) and by Doherty & Popenoe (100). This method is based on asymmetric enzymatic synthesis of anilides by the action of papain on N-acyl amino acids in the presence of aniline; one of the advantages is that the resulting anilides are insoluble. A variation of this method was employed by Huang & Niemann (101), who resolved DL-phenylalanine by carrying out an enzymatic synthesis of acetyl-L-phenylalanyl-paratoluidine. They obtained, thereby, a yield of 82 per cent for the L-form and 77 per cent for the D-form.

C_3.—α,β-Diaminopropionic acid (102) and S-benzylcysteine (103) have

been resolved by the action of acylase-I (Greenstein) upon the corresponding acetyl derivatives.

C_4.—Threonine has been resolved through the brucine salt of the *p*-nitrobenzoyl derivative (104). Tatsuoka *et al.* (105, 106) have resolved methionine by fractional crystallizations of the derivative obtained by the action of ammonium α-bromo-*d*-camphosulfonate on the methyl ester of the amino acid. Fox & Kobayashi (107) have described D and L-diaminobutyric acids separated by the method of Adamson.

C_5.—Baker *et al.* (108) have resolved isovaline by treatment of the N-chloroacetyl derivatives with acylase-I from hog kidney. Proline has been resolved into its optical isomers by the action of amidase from dog kidney on DL-proline amide (109). Berlinguet & Gaudry (110) also used the enzymatic method of Greenstein for the resolution of DL-α-amino-ϵ-hydroxy-*n*-valeric acid by treating the N-chloroacetyl derivatives with acylase-I.

C_6.—DL-leucine has been resolved through various ester derivatives reacting with tartaric acid (111). Birnbaum & Greenstein (103) have extended the method of acylase action to arginine and histidine in order to achieve the resolution of these amino acids through their acetyl derivatives. Lysine has been separated by Emmick (112) into its enantiomorphs by fractional crystallization of their salts with L-glutamic acid. DL-α-amino ϵ-hydroxy-*n*-caproic acid has been resolved by the same enzymatic method as was used with the corresponding derivative of valeric acid (110). Barker (113) states that N-(acetyl or benzoyl-) aspartic acid is racemized less easily than the other acylated amino acids; in order to obtain adequate racemization it is necessary to pass through the intermediary, N-benzoylasparagine. In the conditions of racemization indicated by Du Vigneaud & Meyer, one obtains in this way a racemization of 95 per cent (114).

Determination of Amino Acids

Numerous types of methods for the determination of amino acids, either by "quantitative" chromatography directly on paper, or after separation by paper chromatography and elution, have been proposed this year (32, 115 to 122). Spies (123) has developed a microspectrophotometric method for the estimation of total amino acids. This method, based on the ultraviolet absorption of the copper salts of amino acids, permits the estimation within 3 per cent of from 50 to 500 μM of amino acid; it appears suitable for following the course of protein hydrolysis.

C_2.—Christensen *et al.* (124) have further perfected the already excellent method of Alexander *et al.* (124a) for the determination of glycine. The Van Slyke method for the determination of glycine and glycyl peptides has been improved somewhat by the addition of mercuric chloride to the raction mixture, as proposed by Sluyterman & Kooistra (125).

C_3.—The determination of cystine by phosphotungstic acid has been appreciably improved by Kolb & Toennies (126). Sullivan & Folk (127) have published a method for the estimation of lanthionine based on the

quantitative transformation of this compound into cysteine when it is heated in the presence of a mixture of sodium cyanide and cyanogen bromide.

C_4.—A method for the determination of asparagine and glutamine in plant tissues has been proposed by Butler (128); it consists of paper chromatography of the amides, followed by elution and determination of the ammonia formed after hydrolysis with potassium hydroxide.

A critical study of the nitration reaction according to Kapeller-Adler for the determination of phenylalanine has been made by Beer *et al.* (129). Zalta & Khouvine (130) have developed a new determination for phenylalanine: after nitration under rigorously determined conditions, the product is reduced to an amine by zinc and then diazotized with α-naphthylamine; the AZO compound obtained, whose absorption maximum is at 540 mμ, enables one to determine from 20 to 30 μg. of phenylalanine. Udenfriend & Cooper (131) have made a practical modification of the 1-nitroso-2-naphthol method for tyrosine by using nitric acid to convert the unstable red derivative into a yellow substance which can be extracted with ethylene dichloride or chloroform. Monnier & Besso (132) have proposed a new tryptophan determination based on nitration and polarographic determination of the nitrated tryptophan. Larson *et al.* (133) have developed a microradiochemical method for the determination of thyroxine. The method utilizes the exchange, in solution, of iodine of thyroxine with I^{131}, by this method it is possible to determine 0.1 μg. of thyroxine.

Kraut *et al.* (134) have improved the determination of arginine by the Sakaguchi method by employing the sodium salt of 1-naphthol-8-sulfonic acid. This reagent increases the stability and sensitivity of the reaction. Hunter (135) has described a modification of the colorimetric histidine method, based on the reaction of Knoop. Vukcevic-Kovacevic & Bican-Fister (136) have proposed the determination of histidine, isolated on paper, by a procedure based on the reaction of Knoop, utilizing the resultant coloration for a semi-quantitative determination; histamine does not react under these conditions.

Electrodialysis.—Synge (137), in the course of a study of free amino acids in ray-grass (*Lolium perenne*), described the separation of neutral amino acids from peptides by electrodialysis in an apparatus having four compartments.

Chromatography

Paper chromatography.—Reviews of the various modes of application of paper chromatography to amino acids have been published by Block *et al.* (138), Berry *et al.* (139), and by Jutisz (140). Numerous variations in technique have been described: purification of solutions to be analyzed, either by ion exchange (141 to 144), or by electrolysis (145, 146); the utilization of new solvents (147 to 151), and the role of water in these solvents (152); the introduction of new arrangements (circular chromatography, etc.) (153, 154, 155); and quantitative chromatography.

Chromatography on columns.—The now classic experiments of Partridge and of Moore & Stein have shown that amino acids can be separated from each other by chromatography on ion exchange columns. The technique developed by Moore & Stein as an analytical method has been modified by Hirs, Moore & Stein (156) for use as a method of amino acid preparation. In order to accomplish this, the authors have used volatile buffers as the eluant. The buffers are then easily removed from the eluate by sublimation. For example, the method was applied to the isolation of amino acids from an acid hydrolysate of 2.5 gm. of bovine serum albumin. Four ion-exchange columns were used: one of Dowex 50 (7.5×15 cm.), for separation of basic amino acids; one of Amberlite IR-4B (7.5×15 cm.) for the acidic aminoacids; and two others of Dowex 50 (7.5×120 cm. and 7.5×60 cm.) for the neutral amino acids. Elution was effected by means of ammonium acetate or ammonium formate buffers at pH values of from 3 to 7. The buffers were removed by sublimation at 40° and the amino acids were recovered directly in crystalline form. With the exception of methionine, it was possible to obtain all the amino acids analytically pure with an average recovery of 66 per cent. All were found to be pure L-antipodes, except cystine which underwent considerable racemization in the course of hydrolysis.

Aqvist (157) has adapted the method of Stein & Moore to the isolation of amino acids labeled with N^{15}, starting with biologically synthesized proteins. The hydrolysate from these proteins is first separated into three groups before being chromatographed on Dowex 50. This method can be applied to a hydrolysate corresponding to 25 to 50 gm. of protein. All the known amino acids, with the exception of cystine and tryptophan, can thus be obtained in pure form. Aqvist (157) has also improved the technique of starch chromatography, making it possible to separate the amino acids from a hydrolysate of 300 mg. of protein. This technique has enabled the author to study the fate, in the rat, of N^{15} from labeled amino acids (158). Sluytermann & Veenendaal (159) have also modified the Stein & Moore method of analytical starch chromatography, for use as a preparative method, and have employed it for the separation of glycine, sarcosine, and dimethylglycine.

Continuing their investigations on the use of ion-exchange for amino acid separation, Partridge & Brimley (160) have developed a method for the separation of amino acids from hydrolysates of 200 to 300 gm. of protein. They give as an example the results obtained with commercial ovalbumin, or with the proteins extracted from pressed baker's yeast. The authors use Zeo-Karb 215, Dowex 2 (161), and a sulfonated polystyrene resin prepared in the laboratory. From 280 gm. of ovalbumin, 16 amino acids were isolated with an average recovery of 54 per cent; and from 10 gm. of yeast protein, 13 amino acids were isolated in crystalline form with an average recovery of 60 per cent. Partridge (162) has pointed out the value of ion-exchange resins of the sulfonated polystyrene type, in which the degree of cross-linkage and consequently the pore dimensions can be calibrated at will, for

the separation of amino acids and low molecular weight peptides from polypeptides and proteins.

Brenner & Frey (141) employ chromatography on columns to separate amino acids into three groups: acidic derivatives are retained on an Amberlite IR-4B column and the basic compounds on Amberlite IR-C50. Tabone *et al.* (163) have separated kynurenine from tryptophan on a hydrocellulose column, using as a solvent ethyl alcohol slightly acidified with sulfuric acid. O'Connor & Bryant (164) have made a systematic study of the adsorption of glycine and aspartic acid on a series of synthetic or natural insoluble mineral salts (zinc sulfide, lead sulfide, lead manganate, etc.).

Chromatography of dinitrophenyl amino acid derivatives on columns has also been the subject of some interesting investigations. Middlebrook (165) has developed a technique for the separation of dinitrophenyl amino acids on silica gel columns buffered at pH 6.0, 7.2, and 10.7. He has applied his method to a study of the free amino groups existing in wool. Green & Kay (166) have described a method of simultaneous separation and identification of 16 dinitrophenyl amino acids by chromatography on silica-celite. Mills (167) has achieved the quantitative separation of all the amino acids present in a protein, by the formation of ether-soluble dinitrophenyl derivatives, on a single kieselguhr column; solvents used were mixtures of methylethylketone, chloroform, and water, in variable proportions. Knessl *et al.* (168) also employs either kieselguhr, as a support of the aqueous buffer phase, then considered immobile, or kieselguhr previously treated with a silicone preparation, which thus becomes a support for the organic phase.

Formation of Metallic Complexes

Perkins (169) has carried out potentiometric studies on the stability constants of complexes of a series of α-amino acids with metals of group II. He claims that these metals fall into two categories: Ba, Sr, and Ca, either do not form complexes or form complexes which are strongly dissociated; Hg, Be, Zn, and Cd, however, form complexes which may have very high stability constants.

The affinity of a series of α-amino acids, possessing three ionizable groups, for heavy metal ions, has been systematically measured by Albert (170). The stability constants vary considerably according to the metal. In the case of tyrosine, ornithine, lysine, arginine, and aspartic acid, the metals in question combine through the α-amino and α-carboxylic groups, as with glycine, studied here by way of comparison; with certain α,ω-diamino acids, the metal is combined through the two amino groups. The series: lysine, ornithine, α,γ-diaminobutyric acid, and α,β-diaminopropionic acid, constitutes a transition between these two extremes of behavior. Cysteine and histidine show peculiarities in their mode of union with cobalt.

Li & Doody (171) have carried out polarographic, potentiometric, and conductometric studies of the reaction, with cupric ion, of the following amino acids: alanine, serine, threonine, aspartic acid, glutamic acid, lysine,

and arginine. Kroll (172) has established the constitution of manganous complexes of glycine, leucine, proline, aspartic acid, histidine, and cysteine. He showed, in particular, that at least in certain cases, these complexes can exist in two equilibrium forms. Osterrieth & Hamoir (173) have stated that in neutral and acid solution, methionine forms two complexes with the silver ion: AgM and AgM_2. Their stabilities are practically independent of pH.

Decomposition

Metzler & Snell (174) have effected a catalytic deamination of serine and cysteine, by heating at 100° in sealed tubes in an aqueous solution containing pyridoxal and aluminum, cupric, and ferric ions. The resulting products in the case of serine are pyruvic acid and ammonia; hydrogen sulfide appears in addition to these in the case of cysteine. Andrews & Bruce (175) have shown that cysteine and cystine are decomposed by $6M$ H_3PO_4 acid at 100° with the production of, notably, hydrogen sulfide, sulfur, cysteic acid, ammonia, alanine, serine, and pyruvic acid. Methionine does not react. On the contrary, using 6 M H_2SO_4, cysteine reacts but slightly, cystine is racemized without decomposition, and methionine is demethylated. Tabone *et al.* (176) have demonstrated the formation of kynurenine from tryptophan by heating in dilute alkaline solution. Amino acids, upon heating in cyclic hydrocarbons of varying degree of saturation, such as decalin or tetralin, or organic liquids, such as anethole, safrole, etc., are decarboxylated to form the corresponding amines with yields of from 80 to 97 per cent (177, 178). Meister (179) has developed a method for the preparation of α-keto acids by enzymatic oxidative deamination of a whole series of amino acids. Linstead *et al.* (180) have decarboxylated N-acyl amino acids by anodic oxidation in alcoholic solution; in this way they have obtained the corresponding alcoxyalcoylamines.

Reactions between aldehydes and sugars and amino acids continue to be the subject of various investigations. Akabori & Narita (181) on heating a strongly acid solution of tryptophan, in the presence of benzaldehyde, arabinose, or glucose, have identified, by paper chromatography, serine, alanine, aspartic acid, and 3-(2-aminoethyl)-indol, among the decomposition products of tryptophan. Hannan & Lea (182) have made a study of the changes which take place at room temperature in a mixture of glucose and α-N-acetyl-L-lysine, which had been freeze-dried, and then brought to increasing degrees of humidity. The two constituents react with a complexity which increases with the relative humidity of the medium. Four substances, as yet unidentified, resulting from these reactions, were observed by paper chromatography.

Proctor & Bhatia (183) have studied the decomposition of tryptophan, tyrosine, phenylalanine, and cystine by cathode rays in doses of from 10^5 to 10^6 r.e.p. The amount of each amino acid decomposed, varied exponentially with the dose used. Swallow (184) has demonstrated that gamma rays

decompose cysteine in aqueous solution by starting the following chain reaction:

$$H_2O \rightarrow H+OH;\ H+O_2 \rightarrow HO_2;\ RSH+OH \rightarrow RS+H_2O;$$

$$RS+RSH \rightarrow RSSR+H;\ HO_2+RSH \rightarrow RS+H_2O_2$$

Fixation of Various Radicals on the Amino Nitrogen

Wolff & Berger (185) have succeeded in acetylating L-leucine and L-glutamic acid, without any racemization, and in good yield, with either ketene or acetic anhydride in acetic acid. Their method, however, does not have general application: L-arginine and L-cystine, for example, are either racemized or decomposed.

In order to gain a more thorough understanding of the mechanism of inhibition of enzymes by diisopropylfluorophosphate and tetraethylpyrophosphate, Wagner-Jauregg *et al.* (186) have studied model reactions between these substances or closely related substances and various amino acids and amines. They claim that diisopropylchlorophosphate reacts more strongly than the fluoro derivative, while giving the same reaction products. N-diisopropylphosphorylated derivatives of the following have been obtained in the course of reactions with the chloro derivative, at room temperature in nonaqueous solutions: glycinamide, esters of glycine, of threonine, and of serine; cyclohexylamine and benzylamine. On the other hand, Ashbolt & Rydon (187) have found that, in 0.08 *M* sodium bicarbonate solution at 38°C., the hydroxyl group of tyrosine reacts easily with diisopropylfluorophosphate, but practically does not react with the corresponding chloro derivative. Wilkinson (188) has prepared L-α-γ-dicarbobenzoxyaminobutyric acid from α,γ-diaminobutyric acid; this derivative, on treatment with phosphorus pentachloride, does not give γ-(carbobenzoxyamino) α-carboxyaminobutyric anhydride, comparable to the product obtained from lysine and ornithine, but L-1-carbobenzoxy-3-(carbobenzoxyamino)-pyrrolidone-2, which upon successive hydrogenations gives 3-amino-pyrrolidone-2.

Rao *et al.* (189) has systematically prepared 18 L-N-chloroacetylamino acids. Amination of these compounds produced the corresponding glycyl-L-amino acids, which were subjected to the action of acylase-I and -II. The rates of enzymatic hydrolysis were compared. Farthing (190) has prepared various amino acid carbonates by treatment with sodium carbonate in methanol. With glycine, for example, one obtains a white powder which, after drying with ether, corresponds to the formula $NaO_2 \cdot C(:NH) \cdot CH_2 \cdot Co_2Na$. The amino acids are regenerated by boiling the carbonates in dilute aqueous barium hydroxide. Numerous amino acids can be purified by this method. Furthermore, glycine carbonate, when treated with an acid chloride ($SOCl_2$ or $COCl_2$), forms anhydro-N-carboxyglycine (191).

Miscellaneous Reactions

Karrer & Ehrhardt (192) have obtained optically active amines from amino acids by reduction of their carboxyl groups. Here, as an example, is

the transformation of phenylalanine into the corresponding amine:

$$C_6H_5\cdot CH_2\cdot CH(NH_2)\cdot COOC_2H_5 \xrightarrow{LiAlH_4}$$

$$C_6H_5\cdot CH_2\cdot CH(NH_2)\cdot CH_2OH \xrightarrow{tosylCl}$$

$$C_6H_5\cdot CH_2\cdot CH(NH\cdot tosyl)\cdot CH_2O\cdot tosyl \xrightarrow{LiAlH_4}$$

$$C_6H_5\cdot CH_2\cdot CH(NH\cdot tosyl)\cdot CH_3 \xrightarrow[liq.\ NH_3]{Na} C_6H_5\cdot CH_2\cdot CH(NH_2)\cdot CH_3$$

In a study of the reaction of cystine with hydrocyanic acid, Schöberl *et al.* (193) have suggested the possibility of the formation of the following derivative of 2-aminothiazoline, existing in tautomeric equilibrium:

$$\underline{S{-}C(NH_2){=}N{-}CH(CO_2H){-}CH_2} \rightleftarrows \underline{S{-}C{=}(NH){-}NH{-}CH(CO_2H){-}CH_2}$$

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II. PEPTIDES

A comprehensive review has been published recently, concerning naturally occurring peptides other than those of the anterior lobe of the pituitary (194). Biologically active peptides and proteins of the anterior lobe of the pituitary are treated in another study (195). These natural peptides will, therefore, not be considered here; for reference to these the appropriate review should be consulted.

General Synthetic Methods

General reviews on the various methods, both early and recent, of peptide synthesis have been published by Wieland (196) and Nicholls (197). Several modifications of classic methods have been proposed: Carpenter & Gish (198) have substituted *p*-nitrobenzoxycarbonyl chloride for benzoxycarbonyl chloride as an acylation agent. The compound is more effective than the reagent of Bergmann & Zervas: crystalline derivatives are obtained with leucine, isoleucine, proline, and hydroxyproline, while the benzoxycarbonyl derivatives of the same amino acids are oils. Furthermore, this new reagent is more stable and can be preserved without decomposition for a year when kept cold and dry. Holley & Holley (199) have proposed *o*-nitrophenoxyacetyl and monochloroacetyl chloride as agents for protecting the amino group of an amino acid in peptide synthesis. *o*-Nitrophenoxyacetyl chloride is a stable reagent, which gives crystalline derivatives of the amino

acids in yields comparable to those obtained with benzoxycarbonyl chloride. *o*-Nitrophenoxyacetyl amino acid derivatives, converted to azides, can be used to prepare peptides without racemization. The *o*-nitrophenoxyacetyl group is eliminated as an insoluble lactam after catalytic hydrogenation. Hillmann & Hillmann (200) have studied the removal of acyl groups used for the protection of amino groups during peptide formation; they state that on heating in absolute ethanol in the presence of dry hydrogen chloride, the formyl, acetyl, dichloroacetyl, and *p*-tolyl sulfonyl groups are easily hydrolyzed, without peptide or ester bonds being affected, while benzoyl, *o*-carboxybenzoyl, trichloroacetyl, and phenylacetyl groups are resistant. Removal of the benzoxycarbonyl group from peptides has been achieved by Waldschmidt-Leitz & Kühn (201) by boiling the peptides with hydriodic acid in glacial acetic acid, and by Boissonnas & Preitner (202) by treating the peptides with hydrobromic acid in glacial acetic acid at room temperature; peptide and ester bonds remain intact.

Nicholls (197) has prepared optically active phthalyl amino acid derivatives using either substituted phthalic anhydride, for example 3-nitro or tetrachloro, or by heating the amino acids in a solution of phthalic anhydride in glacial acetic acid; in this way one avoids the racemization which often occurs when phthalic anhydride is fused with amino acids. Nevertheless, Sheehan *et al.* (203) have obtained a stereochemically pure phthalyl derivative of L-phenylalanine, by fusion of a mixture of phthalic anhydride with the amino acid by maintaining the temperature below 150°C. They were able to synthesize in this way, among other compounds, L-phenyl alanylglycine. The optical rotation of the monohydrate, $[\alpha]_D^{28}$, is +84°4 (0.0299 gm. in 1.5 ml. of water), much higher than the previously recognized value of +54°2 in water. These same authors have pointed out the remarkable resistance of the phthalyl group in the course of acid hydrolysis of esterified N-phthalyl-peptides. In order to remove the phthalyl radical from peptides, Schumann & Boissonnas (204) have proposed a new technique which consists of heating the N-phthalyl peptide derivative with an alcoholic solution of phenylhydrazine and a tertiary base such as tri-*n*-butylamine. Yields are of the order of 80 per cent. These same authors have also shown (205) that benzoxycarbonyl groups are resistant to this treatment; this observation is particularly important in the case of peptides such as phthalylvalyl-(δ-benzoxycarbonyl-)ornithyl- etc., having two substituted groups.

Hofmann *et al.* (206, 207) have proposed a new method for obtaining hydrazides of amino acids or acyl peptides. This method consists of preparing the carbobenzoxy hydrazides of amino acids, $H_2N \cdot CH(R)CO \cdot HN \cdot NH \cdot Cbzo$, using carbobenzoxyhydrazine prepared according to Rabjohn (208). This method makes it possible to avoid the direct action of hydrazine on complex peptides. In a derivative of this type, the amino acid can react through its free amino group with the azide or the acid chloride of another

N-acyl amino acid or with the corresponding derivative of N-phthalyl peptides for example. By hydrogenolysis of the peptide obtained, the carbobenzoxy group is eliminated; the peptide hydrazide can then be converted into an azide to permit another coupling. Hofmann *et al.* have used this method for the synthesis of various carbobenzoxyhydrazides of acylated tripeptides.

Among the new methods which have been proposed for peptide synthesis, those which employ mixed anhydrides instead of acid chlorides or azides, have apparently become particularly important. Among mixed anhydrides studied in this regard, those of N-acyl amino acids with chloroformic acid esters have proven especially useful. This method has been developed independently by Wieland *et al.*, by Boissonnas *et al.*, and by Vaughan *et al.* Wieland & Bernhardt (209) have made comparative studies of the behavior of mixed anhydrides formed by carbobenzoxy-N-glycine and various derivatives of mineral or organic acids. The best results have been obtained with isopropyl, isobutyl, and *s*-butyl esters of chloroformic acid. The reactions which take place are the following:

$$\begin{array}{l} \text{Cbzo}\cdot\text{HN}\cdot\text{CH}_2\cdot\text{COOH} + \text{ClCOOR} \rightarrow \\ \text{Cbzo}\cdot\text{HN}\cdot\text{CH}_2\cdot\text{COO}\cdot\text{COOR} + \text{H}_2\text{N}\cdot\text{CH}_2\cdot\text{COOH} \rightarrow \\ \text{Cbzo}\cdot\text{HN}\cdot\text{CH}_2\cdot\text{COHN}\cdot\text{CH}_2\cdot\text{COOH} + \text{CO}_2 + \text{ROH}. \end{array}$$

Wieland & Bernhard have thus prepared the carbobenzoxy di, tri, and tetrapeptides of glycine with yields of 60 per cent or higher. Boissonnas (210), by an analogous method, employing ethyl or methyl chloroformate in the presence of tri-*n*-butylamine, has obtained ethyl phthalyl-glycylglycinate, ethyl carbobenzoxy-DL-alanylglycinate, phthalyl-glycyl-DL-phenylalanine, and phthalyl-glycyl-DL-phenylalanylglycyl-L-leucine, with yields of 65 to 72 per cent. Vaughan (211) and Vaughan & Osato (212) have obtained, in a similar manner, a series of carbobenzoxy or phthalyl substituted, esterified, di- and tripeptides of valine, leucine, phenylalanine, and glycine. As in the experiments of Wieland & Bernhard, the best results were obtained using mixed anhydrides resulting from the action of *s*-butyl and isobutyl chloroformates. Schumann & Boissonnas (205) have employed this technique for a new synthesis of the pentapeptide, L-valyl-(δ-Cbzo) ornithyl-L-leucyl-D-phenylalanyl-L-proline in which the order of amino acids in the chain corresponds to that in gramicidin S. Furthermore, Boissonnas & Schumann (213) have applied this same method to the cyclization of various peptides; in this way they have obtained cyclo-D-leucyl-glycyl-glycine in a yield of 37 per cent. Kenner (214) and Kenner & Stedman (215) have described the synthesis of various peptides, also through the intermediary of mixed anhydrides; in this case sulfuric acid is also used.

Wieland *et al.* (216, 217) have prepared a series of acylthiophenols which they used for the synthesis of various amides and peptides; for example:

$$\text{Cbzo-N-glycyl-thiophenol} + \text{alanine} \xrightarrow{\text{sodium methoxide}} \text{Cbzo-N-glycyl-alanine.}$$

The principle involved here is a transpeptidation analogous to those which take place in enzymatic reactions. Cronyn & Jin (218) have described a method of peptide synthesis through the intermediary of thio amino acids; by this procedure, thiohippuric acid, heated at 90 to 110°C. in the presence of DL-alanine dissolved in dimethylformamide, forms hippurylalanine with a yield of 70 per cent. This method might be compared to that of Sheehan & Johnson (219) in which, operating under different conditions at room temperature, phthaloylthioglycine reacts with glycine methyl ester hydrochloride to produce phthaloylglycylglycine methyl ester.

Several methods of peptide synthesis make use of heterocyclic intermediates. Davis & Levy (220) have employed the derivatives resulting from the combination of 2-thio-5-thiazolidone with the following amino acids: valine, leucine, norleucine, methionine, L-tyrosine, glutamine, and α-amino-isobutyric acid. Petri & Staverman (221) have employed the oxazolidinediones of various amino acids.

Goldschmidt & Wick (222) have described a method based on the condensation of a Cbzo-N-amino acid with a fatty acid isocyanate ester. Wieland & Schäfer (223) have prepared various oligopeptides from esters of several amino acids, by heating them, or even by simply allowing the esters to stand for a period of time.

Synthesis of Specific Peptides

In addition to the peptides which have just been mentioned in regard to the application of general synthetic methods, various other peptides have been obtained. These peptides are classified here in the following order: peptides containing glycine, peptides containing alanine, etc., regardless of the position of the amino acid which serves as the basis of classification.

Peptides containing glycine.—N^{15}-glycyl-glycine, glycyl-N^{15}-glycine, N^{15}-glycyl-glycinamide acetate, glycyl-N^{15}-glycin-amide acetate [Hoberman & Stone (224)]; phenylacetyl glycyl-glycine, phenylacetyl glycyl-diglycyl-glycine, phenylacetyl glycyl-triglycyl-glycine, phenylacetyl glycyl-glycyl-alanine, phenylacetyl glycyl-glycyl-valine, phenylacetyl glycyl-glycyl-methionine, benzoyl glycyl-glycine, benzoyl glycyl-alanine, benzoyl glycyl-methionine, carbobenzoxy glycyl-glycine, carbobenzoxy glycyl-diglycyl-glycine, carbobenzoxy glycyl-β-alanyl-glycine [Süs & Hoffmann (225)]; glycyl-L-serine, L-seryl-glycyl-L-glutamic acid, L-seryl-glycyl-L-serine [Ienan & Fruton (226)]; DL-alanyl-DL-valyl-glycine, glycyl-DL-valyl-DL-alanine [Kühn & Ruelius (227)]; L-cysteinyl-glycine, glutathione [Rudinger & Sorm (228)]; α-L-aspartyl-glycine, β-L-aspartyl-glycine [Le Quesne & Young (229)]; L-glutaminyl-glycine, glycyl-L-glutamine, L-asparaginyl-glycine, glycyl-L-asparagine [Miller & Waelsch (230)]; glycyl-dehydrovaline, glycyl-dehydroleucine, glycyl-dehydroisoleucine [Meister & Greenstein (231)].

Peptides containing alanine.—L-alanyl-L-serine, L-seryl-L-alanyl-L-glutamic acid [Ienan & Fruton (226)]; γ-L-glutamyl-L-alanine [Rowlands & Young (232)]; hydroxy-L-prolyl-L-prolyl-L-alanine [Neumann & Smith

(233)]; various derivatives of DL-alanyl-L-tyrosine [Werbin & MacLaren (234)]. Brand *et al.* (235, 236) in the course of interesting studies on the influence of the structure of peptides on their optical rotation have synthesized the following peptides, free or as hydrochlorides: trialanyl-alanine (LLLL, LDLL, and DLLL); tetraalanyl-alanine (LLLLL); pentaalanyl-alanine (LLLLLL); alanyl-lysyl-alanyl-alanine (LLLL and LDLL); alanyl-lysyl-alanyl-alanyl-alanine (LLLLL and LDLLL).

Peptides containing serine.—Methionyl-serine [Wieland & Schäfer (216)]; poly O-acetyl-serine [Frankel & Halmann (237)]; phenylacetyl seryl-D-penicillamine [Backer & Ollis (238)]. In a significant study, Stoll & Petrzilka (239) have synthesized a series of seryl-leucine and seryl-leucyl-proline derivatives.

Peptides containing cysteine.—Phenylacetyl-L-cysteinyl-D-valine [Holly *et al.* (240)]; L-prolyl-L-cysteinyl-L-tyrosine [Plentl (241)].

Peptides containing methionine.—Cbzo-L-methionyl-L-methionyl methyl ester; Cbzo-L-methionyl-L-methionyl-L-methionyl methyl ester; Cbzo-L-methionyl-L-methionyl-L-methionyl-L-methionine; 1-nitro-5-naphthalene sulfonyl-L-methionyl-L-methionine; carboallyloxy-L-methionyl-L-methionyl-L-methionyl methyl ester [Brenner & Pfister (242)]; methionyl-histidine [Wieland & Schäfer (216)].

Peptides containing aspartic acid.—Several syntheses of DL-or L-aspartic acid polypeptides, in which the aspartyl residues are in α or β linkages, have been described (243, 244, 245); hydroxy-L-prolyl-L-aspartic acid, L-prolyl-L-aspartic acid [Neumann & Smith (233)].

Peptides containing glutamic acid.—Various syntheses of DL- or L-glutamic acid polypeptides, in which the glutamyl residues are in α or γ linkages, have been described (244, 246). γ-L-glutamyl-L-valine, γ-L-glutamyl-L-leucine [Rowlands & Young (232)]; glutamyl-histidine [Plentl (247)]; γ-L-glutamyl-L-tyrosine [Sorm & Rudinger (248)]; hydroxy-L-prolyl-L-prolyl-L-glutamic acid [Neumann & Smith (233)].

Peptides containing proline and hydroxyproline.—Hydroxy-L-prolyl-L-leucine; hydroxy-L-prolyl-L-prolyl-L-phenyl-alanine; hydroxy-L-prolyl-L-prolyl-L-tyrosine; L-phenylalanyl-hydroxy-L-proline; L-prolyl-β-alanine [Neumann & Smith (233)].

Peptides containing lysine.—A high molecular weight polymer of lysine has been synthesized by Becker & Stahmann (249); even though it does not concern a true peptide, it might be interesting to mention the synthesis of biotinyl-ϵ-lysine, or biocytine, by Wolf *et al.* (250).

Peptides containing aromatic amino acids.—D-phenylalanyl-L-phenylalanine; D-phenylalanyl-D-phenylalanine [Vejdelek (251)]; N-acetyl-D-phenylanyl-L-tyrosine; N-acetyl-L-phenylalanyl-L-tyrosine; N-acetyl-L-phenylalanyl-L-diiodotyrosine; N-acetyl-D-phenylalanyl-L-diiodotyrosine; N-acetyl-D-tyrosyl-L-tyrosine; N-acetyl-L-tyrosyl-L-tyrosine; N-Cbzo-L-tyrosyl-L-phenylalanine [Baker (252)].

Miscellaneous peptides.—ε(ε-Aminocaproyl-amino) caproic acid [Van der Want & Staverman (253)].

Chromatography

Brockmann & Musso (254) have made a systematic study of the behavior of glycine peptides in the course of paper chromatography with various solvents. Rydon & Smith (255) have described a convenient method for revealing peptides, by use of chlorine: the chromatogram is exposed to chlorine gas which becomes fixed to the nitrogen; after removal of excess chlorine, the chloro derivatives are revealed by potassium iodide in the presence of starch.

A detailed study of the behavior of many di- and tripeptides in the course of chromatography on columns, has been carried out by Brenner & Burckhardt (256); these authors have employed columns of Amberlite IR-100, Amberlite IR-4B, and a sulfonic resin which they prepared. They claim that the retention of the various peptides depends in part upon the charge on the constituent amino acid residues, and in part upon the number of these residues; glycyl-glycine, for example, is less strongly retained than glycyl-glycyl-glycine; the presence of side chains also influences chromatographic behavior; thus, leucyl-glycine is more strongly retained than glycyl-glycyl-glycine. In addition, the presence of aromatic amino acid residues markedly increases peptide retention and adsorption phenomena become important.

Chemical Structure of Peptides

A recent review by Khorana (257) is concerned with the reactions employed for study of the chemical structure of peptides and proteins. Several interesting methods for the characterization of N-terminal peptide residues, and for successive degradation starting with N-terminal residues, have been described: Leonis & Levy (258) have improved their method based upon reaction of the free amino group with carbon disulfide, making it applicable to milligram quantities of peptide. In the reaction described by Khorana (259, 260), sodium salts of peptides react with alkyl-alkoxy-dithioformates (dialkylxanthates) in aqueous solution at room temperature. This reaction results in the formation of N-thion-carbalkoxy derivatives, which are soluble in organic solvents. After extraction, these derivatives are split by hydrochloric acid in nitromethane into hydrochlorides of amino acids or of degraded peptides, and 1,4-alkylthiazolid-2-5-dione. Mild hydrolysis regenerates the N-terminal amino acid thus split off. Holley & Holley (261) have proposed a particularly gentle method which appears very promising: the N-terminal group of a peptide reacts with fluoro-4-carbomethoxy-2-nitrobenzene; the derivative thus obtained is reduced by catalytic hydrogenation into a 4-carbomethoxy-2-amino-phenyl peptide. This derivative, in aqueous solution for 5 hr. at 25°C. or 15 minutes at 60°C., splits into the amino acid or degraded peptide and a lactam of the N-terminal amino acid;

the lactam crystallizes easily. According to the technique of Wessely *et al.* (262), the peptide is transformed into its carbalkoxy derivative, which, upon heating in dilute alkaline solution, forms a urea derivative; this latter compound, when treated with hydrochloric acid, forms a hydantoin of the N-terminal amino acid and the amino acid adjacent to it. This hydantoin, after extraction with ether, can be converted into an acid chloride, then reduced with sodium borohydride; the resulting compound is hydrolyzed to give the free N-terminal amino acid and the amino alcohol corresponding to the adjacent amino acid.

Among the methods for the characterization of C-terminal residues of peptides and for degradation studies starting from the carboxyl end of the protein chain, is that of Tibbs (263) based upon reactions formerly described by Schlack & Kumpf; a benzoylated peptide is heated with ammonium thiocyanate in the presence of acetic anhydride in glacial acetic acid. One thus obtains a thiohydantoin which may be isolated from the residual peptide by heating in the presence of hydrochloric acid. The hydantoin is subsequently extracted by nitromethane and hydrolyzed with dilute barium hydroxide for 48 hours at 140°C. The amino acid thus liberated is characterized by paper chromatography. Waley & Watson (264) have proposed an analogous method, but employ more gentle conditions; the thiohydantoin is split in a cold slightly alkaline medium, and is hydrolyzed by hydrobromic acid. However, these methods which give satisfactory results with simple peptides, present some serious difficulties when applied to proteins. Khorana (265), utilizing a reaction previously studied by Zetzsche *et al.*, has suggested the treatment of an N-acyl-peptide by di-*p*-tolyl-carbodiimide at room temperature; the product is an acylurea compound, which is split in cold alkalis; the C-terminal amino acid is subsequently regenerated by alkaline hydrolysis. Boissonnas (266) has described a method which employs anodic oxidation of the peptides; paper chromatography of the amino acids liberated on hydrolysis, shows the absence of the C-terminal amino acid from those originally present, when this particular amino acid occurs in no position other than C-terminal. After a partial hydrolysis, it is possible to repeat the operation and characterize the next amino acid, still, of course, with the same reservation. Waldschmidt-Leitz & Gauss (267) have made a systematic study of the action of carboxypeptidase on a series of dinitrophenyl peptides, identifying the C-terminal amino acids, thus liberated, by paper chromatography.

Physical Properties

Leonard & Pasternak (268, 269) have studied the unit cell dimensions of various di- and tripeptides and their hydrates by x-ray spectroscopy. The structure of a poly-γ-methyl-L-glutamate has been the subject of investigations and discussions by Yakel *et al.* (270) and by Cochran *et al.* (271) who agree upon the existence of a helical structure analogous to those already

envisaged by Pauling and his group. In this particular case there is an α-helix comprising 18 residues in five spirals, the β-carbons being in position 2 rather than position 1.

The ultraviolet absorption spectrum of the peptide bond has been studied by Setlow & Guild (272) and by Goldfarb *et al.* (273) between 230 and 200 mμ. In particular, Goldfarb *et al.* were able to determine that each peptide bond has a molar extinction coefficient of 2,500 to 2,800 at 205 mμ. The infrared spectra of a series of homopeptides of glycine and heteropeptides of glycine and leucine, have been studied by Blout & Linsley (274) between 650 and 4,000 cm^{-1}, these measurements being made on the peptides in the solid state. The results show that in the homopeptides of glycine, there is no evidence of nonassociated amino groups; in addition, the presence of strong hydrogen bonds is definitely indicated. In contrast, there are apparently nonassociated amino groups in certain heteropeptides of glycine and leucine. In both types of peptides, in the solid state, some carboxyl groups are found in ionized and others in nonionized form.

Continuing their investigations on the relationships between structure and rotatory power, Brand *et al.* (235, 236) have measured the rotatory power of various peptides previously cited (p. 651). Dissociation constants of peptides containing glycine, alanine, and lysine have been determined by Ellenbogen (275) with the purpose of studying the influence of optical configuration of amino acid residues on the dissociation of ionized groups. In general, it appears that replacement of one of these residues by its optical isomer produces a slight, but nevertheless real, modification of the dissociation of the groups in question.

The total energy liberated on hydrolysis of the peptide bond has been determined by Dobry *et al.* (276, 277) by several techniques; for benzoyl-L-tyrosyl-glycinamide at 25°C., the free energy of hydrolysis is of the order of −420 calories, while the heat of hydrolysis is −1,550 calories. The reaction enthalpy for the formation of a peptide has been calculated by Breitenbach *et al.* (278), from the heat of combustion of constituent amino acids.

Miscellaneous reactions.—Young *et al.* (232, 279) have studied the autohydrolysis, that is the hydrolysis in aqueous solution at 100°, of various glutamic acid derivatives. In the case of α-bonds, the first step is a cyclization, later followed by the liberation of glutamic acid as pyrrolidone carboxylic acid. In the case of γ-bonds, the γ-glutamyl residue takes part in a sort of intramolecular transamination, which leads to the formation of pyrrolidone carboxylic acid and breaking of the peptide bond. In addition they state that, as one would predict, the γ-glutamyl-valine and γ-glutamyl-leucine bonds are more resistant than the γ-glutamyl-alanine bond. Sanger & Thompson (280) have shown that glycyl-valine, when heated with 0.1 *N* hydrochloric acid, undergoes along with hydrolysis, an inversion in amino acid chain order. Sluyterman *et al.* (281, 282) have studied the transformations undergone by diglycyl-glycine methylester and triglycyl-glycine

methylester, maintained at 100°C. for several days, either in the dry form or in anhydrous methanol. They have observed a more or less marked migration of the methyl group with the formation of sarcosyl-peptides, with concurrent polymerizations. Lindenmann *et al.* (283) have shown that phenylthiocarbonyldipeptides, when treated with lead acetate in 70 per cent ethanol, are converted into hydantoin. Kuhn & Ruelius (227), continuing their investigations on the action of diazomethane on peptides, have demonstrated the formation of betaine. From these investigations, they have drawn some interesting conclusions on the dissociation states of amino and carboxyl groups. Upon treatment of various esters or amides of N-carbobenzoxypeptides by lithium aluminum hydride in tetrahydrofuran at 80°C. for 8 to 30 hr., Karrer & Nicolaus (284) have observed that the peptide bond is reduced with the formation of N-substituted amino alcohols. Fels (285), by means of electrophoretic studies, has demonstrated the formation of complexes between certain peptides and trichloroacetic acid. Hannan & Lea (286) have extended to a polylysine, their studies on the reaction of free amino groups of lysine with sugars.

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III. PROTEINS

Preparation

Several general methods for the separation of proteins have been introduced and improvements have been made in some of the methods already in use.

Chromatography on columns.—This method is proving to be extremely fruitful for the separation and isolation, in very pure form, of proteins having similar structures and occurring in mixtures which have not yet been separated by other techniques such as electrophoresis and ultracentrifugation. Proteins may be chromatographed on columns either by partition between two liquid phases, the column serving only as a support for one phase, or by making use of adsorption phenomena or ion-exchange, the column in this case being packed with Amberlite IRC-50, for example, and washed with a buffer at the appropriate pH. It must be remembered that the phenomena which come into action are never simple and that true adsorption takes place to some extent in all such systems. An important systematic

study of the separation of proteins by ion-exchange has been made by Sober *et al.* (287), in which Dowex 50 was employed for the quantitative separation of egg white albumins, various synthetic mixtures of bovine plasma albumins, and human carbon monoxide hemoglobins. These authors consider that the homogeneity of a protein, as established by column chromatography, should be an indispensable criterion of purity. Morawetz & Hughes (288) have studied the interactions between bovine serum albumin and various synthetic polyelectrolytes (polymetacrylic acid, copolymeric metacrylic-vinylpyridine acid, etc.) as a function of pH, ionic strength, the ratio of polymer to protein, and the degree of polymerization of the resin. The results obtained make possible a better control of protein separation by resins and explain some of the difficulties encountered in these separations. Tallan & Stein (289) have subjected lysozyme carbonate from the white of hens' eggs, to chromatography on Amberlite IRC-50 in 0.2 *M* phosphate buffer, pH 7.18; they have shown that this protein gives either a single peak, when freshly prepared, or two peaks, both corresponding to active substances. Martin & Porter (290) have separated a sample of ribonuclease, shown to be electrophoretically homogeneous, into two enzymatically active constituents by partition chromatography on Hyflo-Supercel with a liquid system composed of two phases formed from a mixture of ammonium sulfate, ethylene glycol monoethylether, and water.

Paper chromatography.—Hall & Wewelka (291) have made a theoretical study of the movement of proteins on filter paper. Quastel *et al.* (292, 293, 294) have carried out detailed studies on the separation of several proteins with various solvents: normal and pathological human plasma, cobra venom, and viper venom. A method of two-dimensional paper chromatography and its application to cytochrome-*c* and γ-globulins of human plasma, have been described by Tauber & Petit (295). The protein spots are revealed with eosin and methyl orange. These authors emphasize the fact that each type of protein requires a particular solvent system for its separation; for this reason, paper chromatography, as currently employed, cannot give satisfactory results in the case of complex mixtures such as serum. Robinson & Fehr (296) have developed a quantitative procedure which enables them to separate a mixture of insulin and protamine and to determine each of the components. Paper chromatography has also been used successfully for the separation of various enzymes: phosphoglucomutase and invertase of yeast (297), amylases (298, 299), phosphatases (299); and for separation of plant viruses (300).

Counter-current distribution.—This general method, which seems very promising, has been utilized by Harfenist & Craig (301) for the study of insulin from various sources. With secondary butanol: 1 per cent aqueous dichloroacetic acid as the solvent system, using about one thousand transfers, they have separated into two fractions a sample of insulin which had been considered extremely pure according to the criteria of solubility and electrophoretic and ultracentrifugal behavior. These two fractions show no differences in their amino acid composition.

Salting-out.—Derrien (302) has given details on his method for the es-

tablishment of salting-out curves for proteins. This method has been employed by Pope *et al.* (303) in their study of the heterogeneity of diphtheria toxin and by Steyn-Parvé (304) for the purification of thiaminokinase from yeast. Reid & Jones (305) have fractionated plasma proteins by gradually reducing the concentration of salt in solutions of these proteins by controlled adsorption on Amberlite IR-100 H and IRA-400. Brown (306) has employed systematic salting-out procedures for determining the homogeneity of crystalline catalase from beef liver; in this way he has confirmed the existence of two active constituents.

Electrophoresis.—General reviews on the numerous applications of this method to proteins have been published by Grassmann (307) and Hess (308). In particular, Hess has emphasized criticisms of electrophoresis as a criterion of purity. Electrophoresis of various proteins adsorbed on different types of supports such as wax, paraffin, etc., has been studied by Cumper & Alexander (309). When studied in the classic apparatus of Tiselius, the adsorbed proteins have, in general, the same mobilities as in solution, with the qualifications that the adsorbent surface contain no polar groups and that the proteins be studied in appropriate concentration. Kamal & Turner (310) have studied the homogeneity of bovine thyroglobulin and iodinated γ-globulin by electrophoresis at pH 6.0, 7.5, and 8.3. Brown *et al.* (311) have applied electrophoresis-convection to the separation of γ-globulin and albumin from bovine plasma; the phenomena observed in the course of this separation correspond perfectly to those which are calculated. Hence, this electrophoretic method can conveniently be used for the fractionation of protein mixtures, provided that these mixtures are not too complex. Grassmann & Hannig (312) have described a simple arrangement for paper electrophoresis and a method which makes possible the direct analysis of serum proteins on paper [see also Schneider (313)].

Egg proteins.—Young & Phinney (314) have fractionated the proteins from the yolk of hens' eggs and those from salmon eggs. They have obtained, among others, a lipovitellin from hens' eggs, with an isoelectric point between pH 3.7 and 3.9, and a lipovitellin from salmon eggs, with an isoelectric point between pH 3.4 and 3.5; both proteins are electrophoretically homogeneous and easily denatured in aqueous solution at 20°C. Fraenkel-Conrat *et al.* (315) have prepared several fractions of avidin from the white of hens' eggs. These fractions have been designated avidin A, avidin N A, a complex between avidin A and a nucleic acid, and avidin X A, a complex between avidin A and an acidic substance which has not yet been identified. In any case, each avidin molecule is capable of fixing two molecules of biotin. Conalbumin from hens' eggs has been crystallized by Warner & Weber (316), who, for the purification of this protein, have taken advantage of its ability to form a complex with iron. Ovomucoid, already recognized to be electrophoretically nonhomogeneous, has been fractionated by Bier *et al.* (317) by means of electrophoresis-convection into five components, three of which possess the same antitryptic activity.

ACTH.—The adrenocorticotropic hormone (ACTH) from sheep has been

subjected to several new and increasingly refined purifications; Hess *et al.* (318) have obtained an active preparation, free from 77 per cent of the nitrogenous impurities, by electrodialysis in a three-compartment apparatus. At pH 3.6, all the biological activity passed into the anodic compartment. Nevertheless, Hess *et al.* (319) have shown subsequently that by counter current distribution between 2,4,6-collidine and water, this preparation could be further fractionated into three components, of which a single one, corresponding to 16 per cent of the dry weight of the starting material, contained almost all of the biological activity present. An even superior purification has been obtained by Li (320) and by Geschwind *et al.* (321), by means of successive adsorptions on oxycellulose, either in suspension in a solution of the hormone, or as a chromatographic column. Using the preparation thus obtained, Hess & Carpenter (322), by partition chromatography on kieselguhr with isobutyric acid: 0.2 *N* hydrochloric acid, have isolated a product ten times as pure, with a biological activity of 200 to 400 USP units per mg. Dixon *et al.* (323) have utilized an ion-exchange column to separate a crude preparation of ACTH from hog pituitary into several constituents, only one of which possessed physiological activity. By this method a one hundred fold purification was easily achieved.

Enzymes.—Various enzymes have been obtained in crystalline form: α-amylase from *Bacillus amyloliquefaciens* (324), saccharogenic amylase from *Bacillus subtilis* (325), taka amylase (326), β-amylase from malt (327), pancreatic amylase from hog (328), Z-enzyme from potato (329), and fumarase from hog heart (330).

Other proteins.—Apoferritin has been crystallized by Keil & Poluskova (331), in a form which is practically free of iron. Koechlin (332) has isolated and crystallized from fraction IV-7 of human plasma, a β_1-metal-combining protein, each molecule of which can fix two atoms of iron or copper. Other proteins have been obtained in highly pure form: rhodopsin from cat eye (333), elastin from the aorta (334), growth factor from bovine plasma for *Staphylococcus albus* (335), trypsin inhibitor from lima beans (336), and γ-globulin from rabbit (337). Several studies have also been done on the extraction and purification of muscle proteins: Henrotte (338) has isolated a crystalline protein from carp muscle by electrophoresis; myosin-adenosinetriphosphatase has been prepared from rats by Steyn-Parvé & Gerritsen (339) by a modification of the method of Bailey; Kessler & Spicer (340), have obtained myosin free of actin and lipids from rabbit.

A large number of other proteins has been obtained; however, their degree of homogeneity cannot yet be judged: toxins from *Shigella dysenteriae* (341), diphtheria toxin (303, 342), ricin (343), globulins from various legumes and grains (barley in particular) (344), proteins from almonds (345), epicuticles from wool (346), histones from chicken erythrocyte nuclei (347), Q-enzyme from *Polytomella coeca* (348), and potato phosphorylase (349).

Amino Acid Composition

The amino acid composition of the following proteins has been the sub-

ject of various investigations: salmine (350), clupeins (351), sericin (352), β-lactoglobulin (353), avidin A (354), crotoxin (355), ricin (343), epicuticle from wool (356), crystalline pyrophosphatase (357), crystalline bacterial α-amylase (358), tobacco mosaic virus (353), polyhedral virus from *Bombyx mori* (359), and a capsular virus from *Cacoetia murinana* (359). In addition, certain amino acids have been specifically determined in various proteins: hydroxylysine, found in gelatin and wool, but not occurring in casein, lactalbumin, glycinin, zein, or horse heratin (143); glutamic acid, determined in gliadin (360); and basic amino acids, determined in lysozyme (361). A comparison of the amino acid composition of a particular protein from several different origins has been the subject of several investigations. Proteins studied in this regard were iodinated scleroproteins (362, 363), and especially insulin (364, 365); in the case of beef, hog, and sheep insulin, differences were evidenced only in serine, threonine, glycine, alanine, valine, and isoleucine, according to the study made by Harfenist & Craig (365).

Hydrolysis

Underwood & Deatherage (366) have studied the use of a sulfonated polystyrene resin, Dowex 50, for the hydrolysis of casein and of a protein extracted from coffee. The protein is refluxed in the presence of the resin for 70 hours; in this way one obtains complete hydrolysis and avoids formation of humins. Glutamic acid is converted to pyrrolidone-carboxylic acid, and apparently, destruction of tryptophan is not eliminated. Waldschmidt-Leitz & Zinnert (367) have investigated the use of alkylsulfonic acids of long chain alkyl sulfates for partial hydrolysis of clupein. While hydrolysis by mineral acids follows a fairly continuous course, hydrolysis by decane sulfonic acid, for example, gives rise to a series of plateaus, the position of which depends on the acid concentration. In an attempt to avoid the destruction of tryptophan, Pedersen & Baker (368) tried hydrolysis of casein by SO_2, but were unsuccessful. Desnuelle & Bonjour (369) have studied the kinetics of the hydrolysis of the N-acetyl bond by dilute oxalic acid, using various derivatives as models of structural groups existing in proteins. Elliott (370) has demonstrated that when fibroin is treated with sulfuric acid (nearly anhydrous), the majority of peptide chains attached to serine nitrogen become transferred to the hydroxyl oxygen of this amino acid; this finding is interesting as a confirmation of the scheme proposed by Desnuelle & Bonjour (371) to explain the preferential hydrolysis of serine bonds of proteins. Biserte & Pigache (372) have followed the hydrolysis of wool by boiling 0.1 *N* sulfuric acid, as a function of time. The kinetics of the liberation of amino groups and of arginine from certain proteins by acid hydrolysis has been studied by Roche *et al.* (373).

In order to avoid the destruction, during acid hydrolysis, of dinitrophenylaminoacids in dinitrophenyl-proteins rich in tryptophan, Dickman & Asplund (374) have tried preliminary xanthylation of the proteins; dixanthyl tryptophan is shown to be stable with respect to the acids. The authors have applied their method successfully to lysozyme.

Haworth *et al.* (375) have shown that various proteins (casein and proteins from the peanut and soy bean) become resistant to the action of papain, pepsin, and trypsin, on treatment with formaldehyde in acidic media. However, after elimination of the fraction of formol which is only loosely attached the proteins again become substrates for the action of papain; the resulting products are hydrolyzed by trypsin. The fragments resulting from these successive hydrolyses have been isolated and analyzed; it appears that aspartic and glutamic acids play a fundamental role in formol fixation, while lysine and arginine exercise but a negligible influence.

Harris & Li (376) have subjected insulin to the action of carboxypeptidase and of trypsin. Their results confirm the observations already made by Sanger & Tuppy on the mode of action of trypsin. Furthermore, they have demonstrated that the liberation of alanine from insulin by carboxypeptidase causes only a slight loss of hormone activity.

Currie & Bull (377) have studied the molecular weights of the peptides resulting from pepsin hydrolysis of ovalbumin, as a function of time, pH, temperature, and amount of enzyme. These peptides can be grouped into two large categories, one including those of molecular weight less than 2000, and the other, those of molecular weight above 30,000. In these experiments, pepsin was observed to act as a protease at low pH values, and as a peptidase at a higher pH. There is no proof, however, of the absolute homogeneity of the pepsin which was used.

Degradation

Maurer *et al.* (378) have shown that controlled deamination of crystalline ovalbumin by sodium nitrite produces a soluble and an insoluble fraction, of which they have studied various physical and chemical properties (379). Further, in the course of this deamination, the authors (380) have demonstrated several secondary reactions, such as the introduction of nitroso or diazo groups into phenylalanine and tyrosine residues.

By employing sodium thioglycolate at pH 8.5, Haurowitz & Kennedy (381) have reduced disulfide bonds to thiol groups in serum, albumin, γ-globulin, edestin, and ovalbumin. These disulfide linkages are apparently indispensable to the existence of native serum albumin; in addition, they serve as links between the subunits of γ-globulin. However, in the case of edestin, splitting of these bonds does not change the shape of the molecule.

Cooley & Wood (382) have demonstrated that the sulfur of cystine and a portion of that from methionine can be eliminated from certain proteins by treatment with Raney nickel in an alkaline medium. Casein and ovalbumin, treated in this manner, retain all their other component amino acids. In particular, this method makes it possible to prepare a protein completely free of cystine, but still retaining methionine.

The optimum conditions for the photo-oxidation of β-lactoglobulin in the presence of methylene blue have been determined by Weil & Buchert (383); this photo-oxidation affects first of all the rings of the histidine and

tryptophan residues, and afterwards, that of tyrosine. The oxidation is not accompanied by any rupture of peptide bonds. Morel & Grabar (384), by means of viscosity and osmotic pressure measurements, have shown that the molecular weight of gelatin is considerably reduced when the protein is treated with ultrasonic vibration or with hypobromite; values found by these two methods were not entirely the same.

Denaturation

General considerations of the mechanism of denaturation are discussed by Goldfarb (385). Jirgensons (386) has studied the guanidine and sodium laurylsulfate denaturation of the following proteins: serum albumin, serum γ-globulin, pepsin, lysozyme, arachin, amandin, and Bence-Jones protein, as a function of time, by means of optical rotation and viscosity measurements. Dervichian *et al.* (387) have studied, by means of x-ray scattering, urea denaturation of horse hemoglobin and serum albumin, and of hemocyanin from the snail. Viscosity and diffusion measurements have been used by Chernyak & Pasynskii (388) to study modifications in the form of various proteins in the course of their denaturation by chemical agents. Gibbs *et al.* (389, 390) have investigated the kinetics of ovalbumin denaturation between pH 0.9 and 3.4, at temperatures ranging from 25.0 to 44.4°C. This denaturation proceeds as a first order reaction, and involves suppression of the dissociation of either one or two hydrogen ions of the protein. Urea-denaturation of ovalbumin has been followed by Foster & Samsa (391) with the aid of observations on birefringence of flow.

Christensen (392) has carried out some interesting studies on urea-denaturation of β-lactoglobulin. His observations are in agreement with the hypothesis of Linderstrøm-Lang, namely, that denaturation proceeds according to the mechanism: $N_s \rightleftarrows N_u \rightleftarrows D \rightarrow D_i$, where N_s and N_u represent respectively the stable and unstable forms of native protein, and D and D_i the reversible and irreversible forms of denatured protein. The protein obtained after reversible denaturation behaves exactly as the native protein with respect to trypsin. Denaturation of β-lactoglobulin has also been studied by Larson & Jenness (393) by means of the reactivity of SH groups appearing as a function of time. According to Gorini & Audrain (394), the presence of calcium causes a considerable increase in the denaturation of ovomucoid, rendering the protein easily subject to hydrolysis by trypsin. Zaiser & Steinhardt (395) have studied the kinetics of acid denaturation of horse carboxyhemoglobin. The action of x-rays on proteins has been the subject of various investigations: serum albumin (396), other serum proteins (397), fibrinogen (398, 399), and ovalbumin (400). The action of ultraviolet radiation has been studied by Shugar (401) on lysozyme and by Hink & Johnson (402) on serum albumin. Buzzell & Sturtevant (403) have carried out a thermochemical study of denaturation and inactivation of various samples of pepsin, as a function of pH and temperature.

Gorini & Audrain have demonstrated a protection of serum albumin by

calcium and manganese (404), and of plasmin by zinc (405), against heat denaturation. Hink & Johnson (402) have shown that serum albumin can be protected against ultraviolet denaturation by sodium caprylate.

Interactions between Proteins and Various Substances

Combinations between proteins and various metals have been studied from a thermodynamic point of view by Fiess & Klotz (406). In addition, a whole series of these combinations has been described by Tanford (407), Kacena & Matousek (408), Surgenor & Noertker (409), and Wolff (410); in particular, studies have been made on complexes between serum albumin and silver (411), zinc (412), copper (406), and cobalt (413).

Interactions between collagens and polymetaphosphates have been the subject of studies by Gustavson & Larson (414). Grabar *et al.* (415) have continued their investigations on phosphorylation of gelatin and pepsin. Mihalyi & Laki (416) have studied the iodination of fibrinogen by iodine.

Reversible binding between bovine serum albumin and various fatty acid anions has been demonstrated by Teresi & Luck (417). Shafershtein & Zinova (418) have shown that horse serum albumin ($M=72{,}000$) reacts with sulfosalicylic acid to the extent of 50 moles of acid per protein molecule; the authors thereby conclude that sulfosalicylic acid behaves in this case as a dibasic acid. Friend *et al.* (419) have studied the interaction between dodecylsulfate and bovine serum albumin, and claim that sodium chloride exercises a considerable influence on the nature of the product formed. The substances resulting from the interaction of bovine serum albumin and suramin, heparin, and chondroitinsulfuric acid, and the interaction of ovalbumin and suramin, have been the subject of a systematic study by Gorter & Nanninga (420), with particular emphasis on their electrophoretic behavior. Weber (421) has studied the fluorescence of products formed by the combination of 1-dimethylaminonaphthalene-5-sulfonyl chloride with serum albumin and ovalbumin.

Many investigations have been carried out on protein-dye interaction. Laurence (422) has applied a method based on measurements of polarization and intensity of fluorescent light to the study of the adsorption of a series of dyes by bovine serum albumin. Karush (423) has studied the reversible combination with bovine serum albumin of *d*, *l*, and *dl* forms of dyes derived from methyl red. Interactions between various serum albumins and methyl orange have been investigated by Klotz *et al.* (424) and by Colvin & Briggs (425). In particular, Klotz *et al.* have demonstrated that, at pH 9.2, the affinity of human serum albumin for methyl orange is considerably greater than that of bovine serum albumin, which would indicate that a difference exists in the configuration of the cationic groups of these two proteins. A spectrophotometric study of complexes formed between methyl orange or phenol red and various proteins, horse and human serum albumins among others, has been made by Kusunoki (426), with particular emphasis on the influence of pH and temperature. The fixation of methyl orange and other

anionic dyes by basic proteins such as lysozyme, histones, and protamines, has been studied by Colvin (427). Haurowitz *et al.* (428) have compared the reaction of Congo red with native and denatured ovalbumin. An analogous study has been carried out by Oster (429) with fluoresceins and various proteins; the absorption spectrum of the dye is modified by the presence of native as well as denatured protein in the case of serum albumin, but only by the denatured form of other proteins such as ovalbumin. The interaction between serum albumin and various thiazines has been studied quantitatively by Allenby & Collier (430); the amount of dye bound to serum albumin was found to be only very slightly more than two moles per mole of protein.

Klotz *et al.* (431) have demonstrated that bovine serum albumin, γ-globulin, and trypsin combine only in negligible amounts with trimethylammonium azobenzene. In another study, Klotz *et al.* (432) have shown that at pH values above 7, human serum albumin forms complexes with anions of the type: p,p'-$(ME)_2$—N—C_6H_4—(N_2)—C_6H_4—X^-, where X^- may be SO_3^-, CO_2^-, PO_3H^-, or AsO_3H^-. The results thus obtained have led the authors to develop some interesting considerations on the distances between certain side chains in protein molecules. Braun (433) has shown that fixation of triphenylmethane dyes to myosin and other proteins takes place through SH groups.

Stahmann & Becker (434) have described a new method for the introduction of numerous glycine residues into bovine serum albumin and chymotrypsin; the proteins do not seem to be denatured in this process; in particular, chymotrypsin retains its enzymatic activity. The reaction between casein and D-galactose, 2-desoxy-D-galactose, D-glucosamine, or N-acetyl-D-glucosamine, has been studied by Lea & Rhodes (435). The reaction between glucose and insulin has been studied by Schwartz & Lea (436). Wald & Brown (437) have shown that the SH groups of opsin take part in its combination with retinene.

Inter-protein or protein-peptide combinations have been the subject of various investigations. Burger & Stahmann (438) have demonstrated that synthetic polypeptides of lysine combine with and precipitate tobacco mosaic virus, while such a precipitation does not take place with free lysin. Oncley *et al.* (439) have studied interactions in the following three systems: β-lipoprotein and γ-globulin, insulin monomers, human serum albumin and the homologous equine antibody. These systems were studied either from observations on their solubility or their electrophoretic behavior. Turner & Boyer (440) have investigated the combination of denatured γ-globulin with various proteins, such as ovalbumin, serum albumin, and casein, and with azoderivatives of these proteins. Dobry & Sturtevant (441) have determined that $\Delta H = 0 \pm 1$ cal. per mole of trypsin at 25°C., for the formation of the trypsin-trypsin inhibitor (soybean) complex.

Interactions between proteins are particularly important in the case of muscle proteins. Laki *et al.* (442) have contributed further proof for the reversibility of the stoichiometric combination between actin and myosin.

Kuschinsky & Turba (443) have demonstrated that the formation of actomyosin is not accompanied by a perceptible change in pH. According to Tonomura & Watanabe (444), the interaction between actomyosin and adenosinetriphosphate (ATP) corresponds to the following mechanism: $M + ATP \rightarrow M.ATP \xrightarrow{+ATP} M.(ATP)_2 \rightarrow M^*.(ATP)_2$ (where M^* represents a modified form of actomyosin M). Transformation of M into M^* is not necessarily accompanied by that of ATP into adenosinediphosphate (ADP). Bowen (445) has followed the changes in pH associated with polymerization of actin under the influence of various electrolytes, notably potassium chloride. Furthermore, Kuschinsky & Turba (446) have observed that polymerization of actin is accompanied by an acidification of the medium, while its depolymerization produces an alkalization.

Numerous investigations have been directed toward the study of intermolecular associations of fibrinogen and the transformation of this protein into fibrin. A study of fibrinogen by Ferry *et al.* (447), by means of birefringence of flow, has confirmed the finding that fibrinogen is partially polymerized in extended aggregates when subjected to the action of thrombin in the presence of hexamethylene glycol (448). Koenig & Perrings (449), on the basis of ultracentrifugal and viscosity measurements, have concluded that fibrinogen is a rigidly asymmetric molecule. By means of light scattering measurements, Steiner & Laki (450) have demonstrated that during clotting of fibrinogen, which has a molecular length of 840Å, larger and larger particles are built up through side-by-side and end-to-end associations of the molecules. The relative importance of the two kinds of association depends upon pH. Laskowski *et al.* (451) have studied equilibria in fibrinogen-fibrin transformation. Measurements of sedimentation and viscosity have enabled Ehrlich *et al.* (452) to follow the action of urea on fibrinogen in the presence of thrombin. The influence of thyroxin on coagulation of fibrinogen by thrombin has been investigated by Allegretti *et al.* (453), Laki & Steiner (454) have studied the polymerization of iodinated fibrinogen. Employing birefringence of flow and viscosity measurements, Hocking *et al.* (455) have arrived at the conclusion that fibrinogen consists of rigidly ellipsoidal molecules having a length of 670 Å, and an axial ratio of 18 to 1.

Molecular Weight, Associations, and Dissociations

Several new determinations have been made on the molecular weight of insulin. Tietze & Neurath (456) and Doty *et al.* (458), on the basis of light scattering measurements, Gutfreund (457) from osmotic pressure determinations, and Oncley *et al.* (439), are all in agreement in attributing to insulin a minimum molecular weight of approximately 12,000. On the contrary, Battersby & Craig (459), and Harfenist & Craig (460), by their method of partial substitution, find a minimum molecular weight of 6,000 for beef insulin. Gutfreund (457), Steiner (461), and Doty *et al.* (458), have studied the reversible association of minimum molecular weight units of the hormone. The degree of association of insulin polymers depends upon pH, ionic

strength, and temperature; at pH 2.2, an equilibrium exists between the monomer and the dimer, while at higher pH values, the trimer and higher polymers are formed.

From centrifugation studies on chymotrypsin B, chymotrypsinogen B, and chymotrypsinogen, Smith *et al.* (462) have found each of these proteins to have a molecular weight of 22,500. This is in fairly good agreement with the value of 25,000 determined for chymotrypsinogen by Tietze & Neurath (456). The molecular weight of sheep ACTH has again been determined by Li & Pedersen (463), and found to be 20,000. In acidic media, the hormone dissociates into biologically active fragments. Holmes & Smith (464), by means of sedimentation and diffusion measurements, have determined the molecular weight of fibroin, solubilized in 0.2 *M* potassium chloride, as 84,000. By analogous techniques, Charlwood (465) has calculated the molecular weight of human serum albumin to be 61,500, instead of 68,500 as previously recognized.

The use of the Spinco ultracentrifuge for the study of various proteins has been the object of specific investigations by Taylor (466) and Miller & Golder (467).

Structure and Physical Properties

General reviews on x-ray analysis of protein structure have been published by Pauling (468), Pauling & Corey (469), Mizushima & Shimanouchi (470), and Zahn (471). In addition, the following proteins have been specifically studied in this regard: ribonuclease (472, 473), insulin sulfate (474), hemoglobin (475), horse methemoglobin (476), carbonmonoxyhemoglobin from human fetal blood (477), human serum albumin (478), bovine serum albumin (479), fibroin and sericin (480), and fibrous and globular forms of fibroin (481). The structure of polypeptide helices has been discussed by Huggins (482), and the absence of planar amide groups in the structures proposed by that author has been criticized by Pauling & Corey (483).

McMeekin & Marshall (484) have shown that the specific volume of a protein molecule in solution corresponds satisfactorily to the sum of the volumes of the component amino acids. The partial specific volumes of ovalbumin (0.748), bovine serum albumin (0.734), and of bovine γ-globulin (0.720), in aqueous solution, have been determined by Dayhoff *et al.* (485).

Numerous investigations, notably by electron microscopy, have been made on the physical structure of collagen. A publication by Randall *et al.* (486) includes a review on this subject.

Doty & Steiner (487) have made a valuable study of the relations between the structure of bovine serum albumin molecules and light scattering. Debye & Edwards (488) have studied the phosphorescence of various proteins at the temperature of liquid nitrogen, after ultraviolet irradiation. This phosphorescence is very probably a result of certain amino acids, which, when studied singly under the same conditions, produce luminous emission, generally blue, with lifetimes of 3 sec.

Cheesman (489) has studied the behavior of methemoglobin and serum albumin films at the benzene-water interface. The action of a series of detergents on the spreading of serum albumin, casein, and insulin, has been investigated by Gorter & Boter (490). Buchanan *et al.* (491) have employed dielectric constant measurements in order to determine "bound water" in the following proteins: bovine serum albumin, ovalbumin, lysozyme, gelatin, β-lactoglobulin, and horse methemoglobin.

The solubility of bovine fibrinogen in ethanol-water mixtures, containing from 0 to 8 per cent ethanol, has been determined by Morrison *et al.* (492). Hipp *et al.* (493), working with α-, β-, and γ-caseins, have established titration curves, and made parallel measurements of viscosity and density. The authors attribute variations in the properties observed to differences in the amino acid composition of these proteins.

Absorption Spectra

Elliott (494), on the basis of dichroism observed in an isolated ribonuclease crystal, has concluded that the direction of the folded polypeptide chains is very nearly that of the c axis of the crystal; the amide groups also exhibit a marked degree of orientation. The infrared spectra of myosin, actin, and actomyosin, measured by Morales *et al.* (495), exhibit, among others, a band between 2100 and 2200 cm.$^{-1}$, whereas fibrinogen, considered as a typical protein for comparison, has no absorption in that region. Pasynskii & Pavlovskaya (496) have demonstrated that there is considerable alteration in the infrared spectra of human serum albumin, horse serum globulin, gelatin, and casein, after subjection of these proteins to ultraviolet radiation. Perutz *et al.* (497) have studied dichroism in the ultraviolet spectra of crystals of horse methemoglobin and in an oriented gel from tobacco mosaic virus; the dichroism is attributed to the orientation of tryptophan, tyrosine, and phenylalanine rings, in planes approximately perpendicular to the general direction of peptide chains. A general review on ultraviolet spectra of proteins has been published by Dannenberg (498). Further studies have been made on the ultraviolet spectra of the following proteins: chymotrypsin, insulin, pancreatic trypsin inhibitor, gelatin, clupein sulfate (499), and human collagen (500, 501).

Chemical Structure

A comprehensive review concerning the structure of proteins and naturally occurring peptides has been published by Synge (502). Akabori *et al.* (503) have proposed a new general method for the characterization of C-terminal amino acids of peptides and proteins, involving treatment with hydrazine.

Numerous investigations have been devoted to the determination of the order of amino acids in peptide chains of proteins. The most spectacular is that of Sanger, Thompson & Tuppy (504) who, continuing their studies on

insulin, have shown that the structure of fraction A is as follows: Gly-Ileu-Val-Glu-Glu(—NH_2)-Cy-Cy-Ala-Ser-Val-Cy-Ser-Leu-Tyr-Glu(—NH_2)-

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Leu-Glu-Asp(—NH_2)-Tyr-Cy-Asp(—NH_2). These results, which complete

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our knowledge of the amino acid chains of insulin, were obtained through judicious use of both chemical and enzymatic hydrolyses. Stephen (505) has applied Sanger's methods of performic oxidation and fractionation to a study of the insulin "core," obtained by the action of chymotrypsin on the hormone. This "core" can be considered as having retained its original structure, that is four peptide chains, shorter than those of intact insulin, and joined by disulfide bonds. Chibnall & Rees (506), reducing esterified insulin with lithium borohydride, have demonstrated that the insulin molecule contains eight glutamyl residues, six glutaminyl residues, and four asparaginyl residues located in the peptide chains, and two terminal asparagine residues. The presence of asparagine as a terminal residue in insulin has been confirmed by Harris (507), using carboxypeptidase, and by Jollès & Fromageot (508) by means of lithium aluminum hydride reduction.

After insulin, lysozyme has been the protein most studied in regard to its chemical structure. Thompson (509) and Harris (507), employing carboxypeptidase, have demonstrated that leucine is the C-terminal amino acid on the single peptide chain which constitutes lysozyme. Green & Schroeder (510) have demonstrated the presence of lysine in the N-terminal position, a finding which has been confirmed by Jutisz & Pénasse (511) and by Dickman & Asplund (374). Schroeder (512), by fractionation of the dinitrophenyl-peptides obtained by partial hydrolysis of dinitrofluorobenzene-treated lysozyme, has shown the chain order of the first four amino acid residues to be: Lys-Val-Phe-Gly. Furthermore, Acher *et al.* have isolated and characterized various peptides resulting from partial acid hydrolysis of lysozyme: Arg-Cys, Arg-Asp, Lys-Asp, Lys-Gly (146); Arg-His-Lys, Gly-Tyr, and Tyr-Gly (513).

According to Desnuelle *et al.* (514), chymotrypsinogen is composed of a cyclic protein (contains no free N-terminal amino acid). Its transformation into α-chymotrypsin is accompanied by a splitting of the cyclic peptide, with the appearance of two peptide chains, with alanine and isoleucine as N-terminal amino acids, and, according to Gladner & Neurath (515), tyrosine as one C-terminal residue, and either leucine or isoleucine as the other. Sorm *et al.* (516), however, have found alanine and phenylalanine as the N-terminal amino acids of the two α-chymotrypsin peptide chains.

Fraenkel-Conrat *et al.*, by means of reaction with dinitrofluorobenzene (517), and with phenylisothiocyanate (518), have shown that ovomucoid is composed of a single peptide chain with alanine as the N-terminal amino

acid. Pénasse *et al.* (519), using lithium aluminum hydride reduction, have identified and quantitatively determined phenylalanine as the C-terminal amino acid of this protein.

Anfinsen *et al.* (520, 521) have studied ribonuclease by allowing it to react with dinitrofluorobenzene, and also by treating it with carboxypeptidase. As a result of these experiments, they claim that the protein consists of a single peptide chain, having lysine as the N-terminal amino acid, and valine in C-terminal position. Furthermore they have isolated various peptides after peptic hydrolysis of ribonuclease.

Desnuelle *et al.* (522) have applied the dinitrofluorobenzene reaction to human, bovine, pork, and horse serum albumins, and have shown that in every case the N-terminal amino acid is aspartic acid; Keil *et al.* (523) have confirmed this result with bovine serum albumin. Also by means of the same reaction, Weygand & Junk (524) have characterized aspartic and glutamic acids as the N-terminal amino acids in the "old yellow enzyme." Fraenkel-Conrat & Porter (517), by the same method, have found that conalbumin is formed of a single peptide chain, and avidin of three peptide chains; in each case alanine is the only amino acid in N-terminal position.

Various investigations of the structure of products resulting from the action of thrombin on fibrinogen have been carried out by Bettelheim & Bailey (525) and by Lorand & Middlebrook (526); it now appears to be well established that at least two distinct types of peptides are liberated from fibrinogen when it is clotted with thrombin. By means of the reaction of dinitrofluorobenzene on wool, Middlebrook (527) has been able to characterize seven different amino acids in N-terminal positions with four valine, two alanine, eight glycine, eight threonine, two serine, one aspartic acid, and two glutamic acid residues per molecular weight unit (average $M = 60,000$); the author has discussed the structure of a model representing such a unit.

Fraenkel-Conrat & Fraenkel-Conrat (518) have applied the phenylisothiocyanate reaction to conalbumin, and have thus been able to confirm the existence of alanine as the N-terminal amino acid. Employing the same reaction for β-lactoglobulin, they have shown that glycine and leucine are the N-terminal residues.

Steinberg (528) has verified the finding that ovalbumin does not contain a free amino group belonging to an N-terminal amino acid; but he has shown by the use of carboxypeptidase, that this protein does contain a C-terminal amino acid, alanine. It appears, then, that ovalbumin is not a cyclic protein, as formerly believed, but rather that the amino group of the N-terminal amino acid participates in the bond between the protein and the sugars which are included in the molecule.

The existence and arrangement of SH groups in myosin, actin, and actomyosin, has been investigated by Guba (529) and by Turba & Kuschinsky (530), the latter authors having studied, in particular, the action of heavy metal complexes on properties of these three proteins. Tanford & Roberts (531), on the basis of changes in the ultraviolet spectra as a function of pH

and temperature, have suggested that, in native serum albumin, the hydroxyl group of tyrosine may be hydrogen-bonded; hydrogen bonds, however, are certainly not involved in maintaining the native structure of the protein.

Lowry *et al.* (532) have proposed a method for the measurement of total protein by the Folin reagent, after treatment of the proteins by copper in alkaline medium. Bourbillon (533) has described a method for the estimation of globin, based on the formation of a colored complex between this protein and hematin.

III. Proteins

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AUTHOR INDEX

F

G

L

M

S

U

V

SUBJECT INDEX

G

Q

R

U